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MECHANISMS UNDERLYING THE PROTECTIVE EFFECTS OF cAMP IN CARDIAC FIBROSIS:

ACTIN CYTOSKELETAL REMODELLING AND ROLE OF MKL1/MKL2 AND YAP/TAZ-TEAD DEPENDENT TRANSCRIPTION

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ABSTRACT

Cardiac fibrosis is the main pathophysiological processes contributing to heart failure, affecting 900,000 people in the U.K. Excessive extracellular matrix (ECM) production and increased proliferation of cardiac fibroblasts contributes towards cardiac fibrosis, which increases myocardium stiffness, causing systolic/diastolic cardiac dysfunction and ultimately heart failure. Current treatment options are only partially effective. Hence, a better understanding of the underlying mechanisms is required to help develop novel therapies. In this thesis, the effects of cAMP-signalling on cardiac fibroblast proliferation and migration were investigated.

cAMP signalling, via PKA and EPAC1, inhibited cardiac fibroblasts proliferation but not migration. This was associated with a change in cell morphology, actin remodelling and reduced RhoA-ROCK signalling. Cyclic-AMP also inhibited SRF and TEAD-dependent transcription. Inhibition of SRF resulted from reduced nuclear localisation of MKL1, which involved PKA and EPAC1 signalling. Pharmacological and siRNA-mediated inhibition of SRF and TEAD co-factors MKL1/2 and YAP/TAZ, respectfully, inhibited cardiac fibroblasts proliferation.

cAMP signalling was associated with a reduction in EPAC1 gene expression, which was mediated by both PKA and EPAC1 signalling and dependent on inhibition of actin polymerisation. Promoter analysis identified an EPAC1 promoter TEAD binding-element that was essential for maximal EPAC1 promoter activity. Inhibition of TEAD activity with siRNA gene silencing, dominant negative mutants or pharmacological inhibitors repressed EPAC1 expression. Importantly, a constitutively active YAP mutant rescued EPAC1 promoter activity but not endogenous EPAC1 mRNA levels after cAMP elevation, indicating involvement of additional mechanism. cAMP reduced histone3-lysine27 acetylation at the EPAC1 proximal promoter, which contributed towards EPAC1 repression.

Taken together, these data demonstrate an important role of PKA and EPAC induced actin remodelling and inhibition of MKL-SRF and YAP/TAZ-TEAD in mediating the anti-mitogenic effects of cAMP in cardiac fibroblasts. The data also describes the existence of a negative feedback loop that regulates EPAC1 expression in response to cAMP-induced actin and chromatin remodelling.

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DECLARATION STATEMENT

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of others, is indicated as such. Any views expressed in the dissertation are those of the author. Chapter 5 of this thesis contains data and text that is published in *BBA Molecular Cell Research* (doi.org/10.1016/j.bbamcr.2019.06.013). I confirm that I am the first author of this paper, that the research presented in it is my own work and that I made a major contribution towards the writing of the paper.

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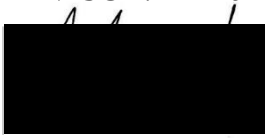
SIGNED:……  …….. DATE: …7 January 2020….

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LIST OF ABBREVIATIONS

6-BNZ-cAMP-AM: N^6- Benzoyladenosine- 3', 5'- cyclic monophosphate, acetoxymethyl ester

8-CPT-cAMP-AM: 8-(4-Chlorophenylthio) adenosine- 3', 5'- cyclic monophosphate, acetoxymethyl ester

A2BR: Adenosine A2B receptor

AA: Amino acid

ABC: ATP- Binding Cassette

AC: Adenyl cyclase

ACE: Angiotensin converting enzyme

ACTA2: Alpha Actin 2

AKAP: A-kinase anchoring protein

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

BD: Biding domain

BrdU: Bromodeoxyuridine

CAK : CDK activating kinase

cAMP : Cyclic 3',5'-adenosine monophosphate

Calm BD: Binding site of calmodulin

CDK : Cycle-dependent kinase

Cx: Connexin

CCN1: Cellular communication network factor 1

ChIP: Chromatin Immunoprecipitation

CKI: CDK inhibitor

CNBD: Cyclic nucleotide binding domain

COOH: C-terminal

COPD: Chronic obstructive pulmonary disease

CRE: CREB response element

CREB: cAMP responsive element binding protein

Ct: Cycle of threshold

CTGF: Connective Tissue Growth Factor

CYR61: Cysteine-rich angiogenic inducer 611

Cyto-D: Cytochalasin-D

DDR: Discoidin domain receptor

DAPI: 4',6-diamidino-2-phenylindole

Db-cAMP analogue: Dibutyryl- cyclic 3',5'-adenosine monophosphate analogue

DEP: Dishevelled, Egr-10, pleckstrin

DMEM: Dulbecco's Modified Eagles Medium

DMSO: Dimethyl sulfoxide

DBPS: Dulbecco's phosphate buffer saline

ECM: Extracellular matrix

EDTA: Ethylenediametetraacetic acid

EdU: 5–ethynyl–2′–deoxyuridine

EPAC: Exchange protein directly activated by cAMP

FAK: Focal adhesion kinase

FCS: Foetal calf serum

FGF: Fibroblast growth factor

FLK: Fetal liver kinase

FSP: Fibroblast specific protein

G1 phase: Gap phase 1

GAF: cGMP-specific PDE, adenylate cyclase and FhIA

GAP: GTPase-activating protein

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GDP: Guanosine-5'-diphosphate

GDI: Guanine nucleotide dissociation inhibitor

GEF: Guanine nucleotide exchange factor

GPCR: G-protein coupled receptor

GTP: Guanosine-5'-triphosphate

HAS1: Hyaluronic Acid Synthase 1

HIF-1α: Hypoxia inducible factor-1α

ISX: N-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide

Lat-B: Latrunculin-B

LIMK: LIM-kinase

LZ: Leucine zipper

M: Mitosis

MAPK: Mitogen-activated protein kinase

MEF2: Myocyte enhancer factor 2

MKL1/2: Megakaryoblastic leukemia ½

MLC: Myosin light chain

MLCP: Myosin light chain phosphatase

MRTF: Myocardin related transcription factors

MMP: Matrix metalloproteinase

MI: Myocardial infarction

MOB1A: Mps one binder kinase activator-like 1A

MRP: Multi Drug Resistant Protein

MTOC: Microtubule organising centre

NF-κB: Nuclear Factor Kappa B

NH_2: N-terminal

PAI1: Plasminogen activator inhibitor1

PAK1: p21-activated kinase

PAS: Per-ARNT-Sim

PBS: Phosphate buffered saline

PDE: Phosphodiesterase

PDGF: Platelet-derived growth factor

PDZ BD: Small COOH-terminal domain that is capable of interaction with proteins having the PDZ domains in their structures

PEDF: Pigment epithelium-derived growth factor

PLN: Phospholamban

PKA: Protein kinase A

PKB: Protein kinase B

pMYPT: Phosphorylated myosin phosphatase targeting subunit

Q: glutamine

RA: Ras associated

RB: Retinoblastoma

REM: Ras exchange motif

Rho: Ras homolog gene family

RhoA: Ras homolog gene family member A

ROCK: Rho-kinase

RT-qPCR: Real-time quantitative polymerase chain reaction

S phase: Synthetic phase

sAC: soluble adenyl cyclase

SAH: S-adenosylhomocysteine

SAP: Scaffold-attachment A/B, Acinus, PIAS

SAV1: Salvador1

SEM: Standard error of the mean

SD: standard deviation

siRNA: Small interfering RNA

SMA: Smooth muscle actin

SRE: Serum response element

SRF: Serum response factor

UVCR: Upstream conserved region

YAP: Yes associated protein

TAD: transcriptional activation domain

TAZ: Transcriptional Co-Activator With PDZ-Binding Motif

TBST: Tris-buffered saline-tween

TIM: Tissue inhibitor of metalloproteinases

TEAD: Transcriptional enhanced associated domain

TGF-β: Transforming growth factor-β

tmAC: transmembrane adenyl cyclase

VEGFA: Vascular endothelial growth factor A

WAVE: WASP family verprolin-homologous protein

WASP: Wiskott-aldrich syndrome protein

ZYX: Zyxin

CHAPTER 1:

INTRODUCTION

1.1 Normal cardiac interstitium

In the adult mammalian heart, ventricular myocytes are arranged in layers of 3 to 5 cells thick, where neighbouring layers are mechanically and electrically coupled together but adjacent layers are separated by clefts with little cell-to-cell coupling (Leonard, Smaill et al. 2012). Layers of myocytes are separated by interstitial collagen deposited by interconnected networks of fibroblasts. Myocardial tissues consist of several different cell types, such as: myocytes; cardiac fibroblasts; endothelial cells; and vascular smooth muscle cells, although most of the cells consist of myocytes and cardiac fibroblasts (Souders, Bowers et al. 2009). Cardiac myocytes and cardiac fibroblasts cooperate to maintain the electrical and contractile nature of a mammalian heart. In addition, cardiac fibroblasts not only preserve the three-dimensional structure of the heart, but they also contribute to the development of the heart, angiogenesis and responses to tissue injuries, which will be discussed in detail later (Camelliti, Borg et al. 2005, Souders, Bowers et al. 2009).

1.2 Heart failure

Heart failure is a condition in which the heart is not able to pump enough blood around the pulmonary or systemic circulation to support normal functioning of the organs. It is characterised as a progressive disorder, which originates from myocardium insults (e.g. infarction, infection, pressure overload, volume overload) or disruption to myocardial contraction (e.g. owing to arrythmia) that prevent the heart from pumping blood efficiently (Mann and Bristow 2005). Over 500000 of the UK population are living with heart failure and its risks increase with aging. Major causes of heart failure include hypertensive heart disease and injuries caused by myocardial infarction (MI) as a result of ischaemic heart disease (Leonard, Smaill et al. 2012). The development of HF can be linked to continuous pathophysiological alterations in the geometry of the heart chambers (e.g. dilatation) and myocardium (e.g. wall thinning, increased stiffness) which are associated with progressive reorganisation of the cardiac extracellular matrix (ECM) (Mann and Bristow 2005). Excessive deposition of ECM produced by cardiac fibroblasts, causes stiffness, which reduces myocardial elasticity (i.e. compliance), leading to diastolic dysfunction, and contractility, leading to systolic dysfunction.

1.3 Cardiac fibrosis

Cardiac fibrosis can occur after any kind of myocardial insult, such as: inflammation (e.g. infection or autoimmunity); MI; and aging-related arterial stiffness (Ivey and Tallquist 2016). The main cell type implicated in the fibrotic remodelling process is the cardiac fibroblast. While defining and studying the behaviour of cardiac fibroblasts is crucial for developing methods to decrease their

negative effects, there is still much uncertainty about their unique gene expression profile, functions and origins (Ivey and Tallquist 2016).

Cardiac fibroblasts originate from mesenchymal cells. They are defined as non-vascular, non-inflammatory and non-epithelial cells. They make an abundant population of cells in the myocardium, responsible for 60-70% by numbers or approximately 20% by volume (Camelliti, Borg et al. 2005, Baudino, Carver et al. 2006). Even though cardiac fibroblasts share similar structural and ECM regulating functions with the fibroblasts of other organs within the body, they have a different behaviour towards certain stimuli, which implies organ specificity (Brown, Ambler et al. 2005).

1.4 Mechanisms underlying cardiac fibrosis

Cardiac fibrosis can be summarised in three different phases: the initiating phase; the effective phase; and the amplificative phase. A study carried out in 2014 demonstrated that, following stimulation, the levels of pro-fibrotic growth factors are increased, which, in turn, trigger the fibrotic response (Kong, Christia et al. 2014). In the next phase, these circulating pro-fibrotic growth factors bind to their specific receptors, which activate specific pathological transcription factors and signalling pathways such as: Protein Kinase B (PKB); Mitogen-Activated Protein Kinases (MAPK); and Nuclear Factor Kappa B (NF-κB). These pathological activations induce the transformation of cardiac fibroblasts to myofibroblasts, which respond by synthesizing ECM components (e.g. collagen 1, collagen 3 and matrix metalloproteinases (MMPs)) or by proliferating, migrating and expressing α-smooth muscle actin (α-SMA), as well as angiotensin II receptors and angiotensin converting enzyme (ACE) (Souders, Bowers et al. 2009, Ma, Yuan et al. 2018). Furthermore, pro-fibrotic transcription factors control the production and secretion of pro-fibrotic growth factors (e.g. transforming growth factorβ, TGF-β) by cardiac fibroblasts and other cells (including cardiomyocytes and inflammatory cells) in the myocardium. Finally, these pro-fibrotic growth factors can form and regulate positive feedback mechanisms and further amplify the extent of the cardiac fibrotic response (Kong, Christia et al. 2014, Frieler and Mortensen 2015, Ma, Yuan et al. 2018).

1.5 Cellular effectors of cardiac fibrosis

Since adult mammalian cardiomyocytes have insignificant regenerative capacity, acute cardiomyocyte death (following acute MI or any cardiac insult)initiates an inflammatory reaction, which eventually leads to the replacement of dead myocardium with collagen scars by cardiac fibroblasts (Kong, Christia et al. 2014). In addition, other injurious stimuli, such as pressure

overload and myocardial inflammation, may trigger profibrotic signalling pathways even when cardiomyocyte death is absent. Several different cell types could also play essential roles in the fibrotic remodelling of the heart, either directly by stimulating the production of matrix proteins from fibroblasts, or indirectly via the secretion of fibrogenetic mediators e.g. by mast cells, lymphocytes and macrophages, cardiomyocytes or vascular cells. In all of the aforementioned conditions, cardiac fibroblasts are differentiated into secretory and contractile cells (known as myofibroblasts) by TGF-β1; and these myofibroblasts are associated in the cardiac fibrotic remodelling and cardiac healing (Kong, Christia et al. 2014). Myofibroblasts are activated fibroblasts differentiated from endogenous fibroblasts, stem cells and infiltrating pericytes. They are different from unstimulated fibroblasts because they express more contractile proteins (such as smooth muscle α-actin) and are able to secrete different factors, such as platelet-derived growth factor (PDGF) and stem cell factors (Powell, Mifflin et al. 1999).

1.6 Marker proteins and the origin of cardiac fibroblasts

No cardiac fibroblast-specific molecular marker has been reported yet. Previous results were obtained using co-labelling with panels of antibodies that can identify cardiac fibroblasts (Ma, Yuan et al. 2018). For example, vimentin, a constituent of the intermediate-filament family of proteins has been used to identify the purity of isolated cardiac fibroblasts *in vitro*. However, endothelial cells and smooth muscle cells can also express it (Mork, van Deurs et al. 1990, Ivey and Tallquist 2016).

Once differentiated and activated, myofibroblasts can express α-SMA. As a result, α-SMA can be used to recognise cardiac myofibroblasts, but α-SMA can also be expressed in other cell types, such as vascular smooth muscle cells (Weber 1997, Ivey and Tallquist 2016). In addition to α-SMA, fibronectin containing extra domain A (ED-A) is also highly expressed in myofibroblasts (Santiago, Dangerfield et al. 2010) and deposited in ECM (Dobaczewski, Bujak et al. 2006). However, it could also be co-localised in endothelial cells (Chang, Chen et al. 2004) and macrophages (Doddapattar, Gandhi et al. 2015).

Moreover, Fibroblast Specific Protein (FSP1) is proposed as a specific marker for cardiac fibroblasts, but an investigation reported that cells expressing this protein were a small subset of cardiac fibroblasts (Moore-Morris, Guimaraes-Camboa et al. 2014, Ivey and Tallquist 2016).

Another protein that has been reported to be a specific marker of cardiac fibroblasts is known as Periostin. It is a secreted protein and is linked with cell adhesion (Ivey and Tallquist 2016). A study carried out in mice reported that this could be a specific marker expressed mostly by activated

cardiac fibroblasts following an injury to the heart. However, another study carried out in mice demonstrated that most Periostin is secreted and then deposited extracellularly and only a tiny portion can be spotted intracellularly (Snider, Hinton et al. 2008). This could limit the utility of this protein in the study of cardiac fibroblasts (Kanisicak, Khalil et al. 2016). A study carried out in rats and mice reported that immunostaining of another protein known as Discoidin Domain Receptor (DDR) 2, which is a tyrosine kinase receptor, can only label a small proportion of the cardiac fibroblasts (Banerjee, Fuseler et al. 2007, Ivey and Tallquist 2016).Further investigation on specific markers of cardiac fibroblasts would be of interest to unveil the mechanism of cardiac fibrosis.

Another significant debate in the study of cardiac fibroblasts is their origin (Ma, Yuan et al. 2018). The traditional belief is that cardiac fibrosis is the result of the proliferation and migration of resident cardiac fibroblasts. This phenomenon is supported by an investigation carried out in 2017 where the authors demonstrated that TGF-β induced the proliferation and migration of isolated mice cardiac fibroblasts (Ma, Yuan et al. 2017). Moreover, an investigation carried out in mice showed that pressure overload could be an important driving factor for the activation and proliferation of resident cardiac fibroblasts (Moore-Morris, Guimaraes-Camboa et al. 2014). On the other hand, this concept has been challenged by the following discoveries: cardiac fibroblasts quickly transform into cardiac myofibroblasts even in the absence of stimuli *in vitro;* and proliferating cardiac fibroblast-like cells predominate in the areas close to the blood vessels, suggesting that, they can migrate from blood circulation (Ma, Yuan et al. 2018). Moreover, another investigation carried out in mice cardiac fibroblasts demonstrated that endothelial cells can also contribute to the total population of cardiac fibroblasts (Zeisberg, Tarnavski et al. 2007). The authors used FSP1 to identify cardiac fibroblasts and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1) as the marker for endothelial cells, although TIE1 could identify immune lineages as well. To add to the complexity, other investigations that used different genetic markers, such as Fetal Liver Kinase 1 (FLK1) and VE-Cadherin, reported limited input of endothelial cells to the total population of cardiac fibroblasts (Alva, Zovein et al. 2006). Further investigations focusing on the contribution of the endothelial-cell-derived cardiac fibroblasts would be of interest (Ma, Yuan et al. 2018).

1.7 Physiological functions of cardiac fibroblasts

There are lots of studies highlighting the prominent roles of cardiac fibroblasts to support and maintain physiological cardiac development, structure and function (Frangogiannis 2012, Deb and Ubil 2014, Travers, Kamal et al. 2016).

Normally, Cardiac fibroblasts are constantly exposed to mechanical signals, and proper regulation of these mechanical stimulations maintain normal cardiac function (Catalucci, Latronico et al. 2008). Furthermore, mechanical load results in a significant enhancement of ECM components, ECM specific receptors, growth factors and cytokines (Butt and Bishop 1997, Souders, Bowers et al. 2009).

Moreover, there is evidence that cardiac fibroblasts are involved in the preservation of the three-dimensional structure of the heart via the autocrine and paracrine action of secreted factors and the direct cell to cell interaction (Souders, Bowers et al. 2009).

1.7.1 Role of cardiac fibroblasts during heart development

In contrast to the well described roles of adult cardiac fibroblasts, there is not much known about the roles of these cells during heart development. An experiment conducted on mouse embryonic cardiac fibroblasts suggested that they could form a fibrous skeleton which would provide structural support for the cardiomyocytes (Leda, Tsuchihashi et al. 2009). Moreover, co-culture experiments suggested that embryonic cardiac fibroblasts could promote cardiomyocyte-genesis during heart development, an effect which required fibronectin and collagen 3 (Leda, Tsuchihashi et al. 2009).

1.7.2 Role of cardiac fibroblasts in angiogenesis

The involvement of fibroblasts in angiogenesis was first reported back in 1993, where it was demonstrated that mouse embryo fibroblasts promoted formation of capillary-like tubes by endothelial cells (Montesano, Pepper et al. 1993), In addition, *in vitro* and *in vivo* experiments have unveiled the crucial role of cardiac fibroblasts in angiogenesis during development and disease stages. Their role in this process has been reported to be via the synthesis and accumulation of ECM, growth factors and cytokines (such as Interleukins 1 and 6), all of which would allow them to control their environment through autocrine and paracrine signalling. Growth factors secreted by fibroblasts include Fibroblast Growth Factor 2 (FGF2) and Vascular Endothelial Growth Factor A (VEGFA) (Murakami and Simons 2008), both of which can act on endothelial cells and have got important roles in neovascularisation and coronary collateral formation in order to support the blood supply to myocardium after injuries (Souders, Bowers et al. 2009). In contrast, it has been reported that, Pigment Epithelium-Derived Growth Factor (PEDF), which is expressed in both cardiac myocytes and cardiac fibroblasts, opposes the effects of VEGFA in angiogenesis *in vitro* (Rychli, Kaun et al. 2010).

These studies underline the pro and anti-angiogenic effects of cardiac fibroblasts in the sprouting of blood vessels and imply that proper regulation of these factors is vital for cardiac angiogenesis and vascular development (Souders, Bowers et al. 2009).

In addition to the growth factors secreted by cardiac fibroblasts, other factors are crucial in angiogenesis, such as MMPs and Tissue Inhibitors of Metalloproteinases (TIMPs). The MMPs exert their effects in part by degrading the ECM, promoting endothelial cell migration and proliferation. They can also control endothelial cell adhesion and survival leading to the activation or inhibition of vascularisation. On the other hand, an experiment carried out in mice in 2003 by Hamano and co-workers demonstrated that the cleavage of MMP-9 generates tumstatin (an angiogenesis inhibitor fragment of collagen 4) which plays an important role in the inhibition of pathological angiogenesis (Hamano, Zeisberg et al. 2003). Like MMPs, TIMPs can have pro and anti-neovascularisation effects too. In a study carried out by Yamada and colleagues in mice, it was shown that, TIMP-1 deficient mice showed slower angiogenesis in their retina (Yamada, Tobe et al. 2001). In another study, it was observed that, exogenous TIMP-1 promoted the formation of new blood vessels. Moreover, a mutant version of TIMP-1 did not induce angiogenesis. It was also demonstrated that the activity of TIMP-1 is dependent on the activity of MMPs (Liu, Chen et al. 2008).

As mentioned before, TIMPs can inhibit angiogenesis at different stages which could be mediated by either the decline in the expression of MMPs or the reduction in their activities (Lambert, Dasse et al. 2004).

Taken together, these studies demonstrate the significance of fibroblasts in the regulation of angiogenesis.

1.7.3 Role of cardiac fibroblast in electrical signalling

Cardiac fibroblasts have other intriguing roles in addition to angiogenesis. Due to their high membrane conductae, they can be good conductors of electrical signals A study carried out in rats in 1997 highlighted the importance of gap and cell junctions through connexins (Cx) in the maturation of electromechanical performance the mammalian heart. (Angst, Khan et al. 1997). Later on, it was demonstrated that cardiac fibroblasts have the ability of making network of cells that are connected to the ECM via integrins, to each other through specific cadherins and to cardiac myocytes by different types of receptors including Cx (Banerjee, Yekkala et al. 2006). A year later, Chilton and co-workers studied the intracellular coupling in co-cultured adult rabbit ventricular myocytes and myofibroblasts. This was examined by tracking the flow of a fluorescent dye. Their

investigation demonstrated that once ventricular myocytes were preloaded with the dye and co-cultured with the myofibroblasts for about a day, the fluorescent dye could be detected in about half of the population of myofibroblasts. To further confirm it, they also treated their co-cultured cells with gap junction uncoupler, which significantly decreased the flow of the dye. Furthermore, their immunostaining unveiled the expression of Cx43 in their co-cultured cells. Their study demonstrated that stimulation of adult rabbit ventricular myocytes can have effects on the intracellular calcium ions (Ca^{2+}) of the co-cultured myofibroblasts most probably through Cx43 (Chilton, Giles et al. 2007). Another study carried out in adult mouse cardiac fibroblasts identified the Cxs involved in gap junctions between them. Using Quantitative real time-Polymerase Chain Reaction (qPCR) and Western Blotting, they showed the presence of proteins and mRNAs of Cx40 and Cx43. Moreover, their analysisshowed that cardiac fibroblasts are functionally coupled together to form an extensive coupled cell network that could link different regions of myocytes that would normally be electrically isolated by connective tissues (Louault, Benamer et al. 2008).

Baudino and co-workers used *in vitro* cell-cell interaction assays to further confirm the communication between cardiac fibroblasts and myocytes via the formation of tight cell to cell junctions (Baudino, McFadden et al. 2008).

In addition to gap junctions, the ion channels of cardiac fibroblasts play an intriguing role in signalling; and it has been shown that abnormalities in them may result in cardiac arrhythmia and dysfunction (Shibukawa, Chilton et al. 2005). An investigation carried out on adult rat cardiac fibroblasts and myofibroblasts highlighted the importance of one of these ionic channels (inwardly rectifying potassium channels), which play important roles in the regulation of resting membrane potential, survival, proliferation and the contractility of cardiac fibroblasts. It was concluded that these channels are activated by the electrical coupling (contact) between the cardiac fibroblasts and cardiomyocytes via gap junctions (Chilton, Ohya et al. 2005).

1.7.4 Roles of cardiac fibroblasts in wound healing

One of the crucial roles of cardiac fibroblasts is to heal wounds after any type of injury to the heart. They play central roles in various aspects of wound healing, from the deposition of ECM, to the formation of new blood vessels at the site of the injury and the maturation of scar (Deb and Ubil 2014). A study carried out on mouse hearts suggested that after MI injury, cardiac fibroblasts rapidly differentiate into their active forms (myofibroblasts) and proliferate (Christia, Bujak et al. 2013). The highest number of myofibroblasts was achieved within 3 days of ischaemia-reperfusion injury. 28 days after ischaemia-reperfusion injury, the number of the cardiac fibroblasts decreased by 90%

compared to its peak, indicating the dynamic feature of the cardiac fibroblasts at the site of the injury (Christia, Bujak et al. 2013). Following an injury to the myocardium, activated cardiac fibroblasts express proteins necessary for contraction (such as: α-SMA) and secrete collagens that are needed for the structural integrity of the heart (Deb and Ubil 2014). These early repair responses from activated cardiac fibroblasts are crucial in wound healing following a cardiac injury as it has been reported that interruption in this process by loss of function experiments could result in downregulation of cardiac performance and retarded wound healing (Duan, Gherghe et al. 2012).

In the later stages of wound healing, the scar gradually contracts, collagen fibres at the site of the injury cross-link together, which increases the tensile strength of the scar and its surface area gets reduced. It has been suggested that, myofibroblasts (which express contractile proteins) contribute to this process, which is known as scar thinning (Deb and Ubil 2014). Scar thinning could lead to adverse changes in ventricular chamber geometry and compliance, would increase the haemodynamic burden on the remaining viable myocytes and over time could result in the development of congestive heart failure (Deb and Ubil 2014).

1.8 Pathological functions of cardiac fibroblasts

As mentioned earlier, cardiac fibrosis is defined by an overall accumulation of ECM in the myocardium and is an essential element of most cardiac pathologies. It develops when excessive amounts of ECM is produced by myocardial fibroblasts in order to form a scar tissue and as a result of this normal degradation of ECM is disrupted (Berk, Fujiwara et al. 2007). Figure 1.1 summarises the phases of cardiac fibrosis.

As mentioned earlier, heart is made of muscles referred as myocardium. The laminar architecture of the myocardium is characterised by complex networks of ECM proteins containing mainly fibrillar collagen (Kong, Christia et al. 2014). The half-lives of mature fibrillar collagens are between 80-120 days which shows their highly stability. The homeostatic control of collagen in a normal heart is mainly controlled by the cardiac fibroblasts and requires constant synthesis and degradation of matrix proteins. The consequence of any disturbance in the metabolism of collagen would be catastrophic: it could induce functional and structural abnormalities in the heart (Kong, Christia et al. 2014). After MI, cardiac fibroblasts proliferate and migrate faster and deposit more ECM in order to prevent cardiac rupture. However, their protective mechanism could also have deleterious and negative side effects which includes permanent change in the shape of the heart (especially ventricles) and reduced electrical transduction.

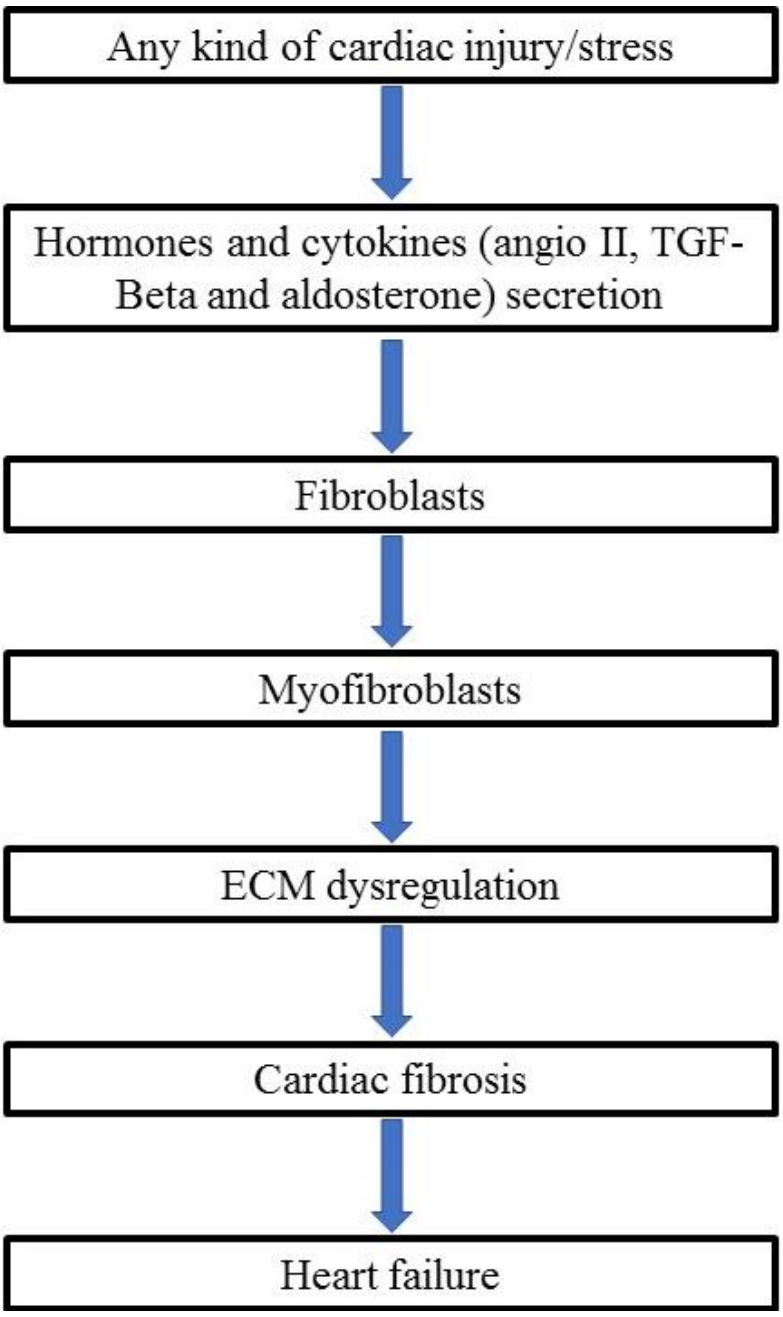


Figure 1.1: Pathogenesis of cardiac fibrosis

Any kind of cardiac injury would stimulate the production of specific hormones and cytokines. This would promote the activation of cardiac fibroblasts to cardiac myofibroblasts. Once cardiac fibroblasts are activated, they proliferate and migrate which would cause the excessive production of ECM. This would lead to cardiac fibrosis and eventually heart failure. Angio II: angiotensin II; ECM: Extracellular matrix; TGF-β: Transforming growth factor-β (Leask 2010, Reilly 2015).

1.8.1 Cardiac fibrosis enhances stiffness of the heart

Irrespective of the initial cause, any kind of injury to the heart provokes sustained cardiac fibrotic responses, which results in an irregular heart architecture and impairs its normal function (Ma, Yuan et al. 2018). In 1988, it was reported that the stiffness in the adult rat myocardium is related to the fibrosis. In addition, an investigation carried out in 1989 on intact rat myocardium suggested that cardiac fibrosis associated with myocyte death has unique morphological features involving fibrillar collagen, which, because of its structure, location and alignment, could lead to an enhanced myocardial stiffness (Carroll, Janicki et al. 1989). Moreover, cardiac fibrosis would cause the slippage of cardiomyocytes resulting in a decrease of the number of cardiomyocytes layers in the left ventricle (Kong, Christia et al. 2014). In 1994, an experiment conducted by Beltrami *et al* in patients suggested that myocardial fibrosis resulted in the expansion of left ventricle and reduction in the ventricular mass-to-chamber volume ratio, implying left ventricular dilation (Beltrami, Finato et al. 1994). Later on, it was suggested that continued deposition of ECM and stiffness of the heart could impair the contractile behaviour of the heart muscles (Weber 1997).

1.8.2 Cardiac fibrosis impairs the conduction of electrical impulses in the heart

Cardiac fibrosis impairs the coordination of excitation-contraction coupling in the myocardium, which would result in the disruption of systolic and diastolic functions (Janicki and Brower 2002). On the other hand, the breakdown of ECM via the action of MMPs (enzymes capable of the degradation of ECM components) has been reported to induce left ventricular enlargement (Iwanaga, Aoyama et al. 2002).

The severe outcomes resulting from the impairment of the collagen network in a fibrotic heart could be due to different mechanisms. It has been reported that fibrillar collagen helps to sustain normal myocardial systolic performance by enabling transduction of cardiomyocyte contraction into the myocardial force development. An impairment would induce an imbalance and uncoordinated contraction of cardiomyocyte bundles (Baicu, Stroud et al. 2003).

Moreover, interactions between collagen and laminin with their receptors could have an essential role in the homeostasis of cardiomyocytes (Wang, Hoshijima et al. 2006). In the same investigation, it was also suggested that mutations in laminin alpha4 cause abnormalities in the structure of cardiovascular ECM, which could lead to insufficient supply of oxygen to the myocardium and cause ischaemia (Wang, Hoshijima et al. 2006), illustrating the connection between matrix network and the structural integrity of the heart (Kong, Christia et al. 2014).

Furthermore, cardiac fibrosis is thought to impair anisotropic conduction, slowing down the velocities of electrical conductions and generating re-entry circuits (Khan and Sheppard 2006, Kong, Christia et al. 2014). More than a decade ago, it was reported that, patients who suffer from chronic Atrial Fibrillation exhibit higher levels of myocardial interstitial fibrosis compared to the control group (Tagawa, Higuchi et al. 2001, Corradi, Callegari et al. 2004). Later, a study carried out by Verheule *et al* suggested that, atrial fibrosis can be a sufficient factor to induce atrial fibrillation in transgenic mice overexpressing TGF-β. Hence, atrial fibrosis could be a significant contributor to atrial fibrillation, even in the absence of other proarrhythmic factors (Verheule, Sato et al. 2004).

In addition, as fibrosis develops, myocardial bundles are separated, which could induce a disruption in the transverse conduction an lead to arrhythmia by atrial fibrillation (Spach, Miller et al. 1982). An investigation in dog hearts confirmed that fibrosis could interfere with conduction in the atria which induced atrial fibrillation (Li, Fareh et al. 1999).

1.9 Potential therapeutic target for cardiac fibrosis

Following MI, the main reasons for the development of cardiac fibrosis are the differentiation of cardiac fibroblasts to cardiac myofibroblasts, their proliferation and their migration (Fan and Guan 2016). Consequently, controlling the differentiation of cardiac fibroblasts, their proliferation and migration rates could be crucial to inhibit cardiac fibrosis. As previously reported, different growth factors and cytokines (TGF-β, angiotensin II and PDGF) play important roles in the differentiation process. Therefore, current therapies (either systematic or localised) are mainly focused on decreasing the secretion of these growth factors and cytokines which in turn would retard the proliferation and migration rate of these activated cardiac fibroblasts (Reilly 2015, Fan and Guan 2016). Some of these current therapies are explained in more details in the next section.

1.9.1 Agents that impact the renin angiotensin system (RAS)

Several investigations highlighted the important role of RAS in the pathogenesis of cardiovascular diseases (Sadoshima 2000, Mezzano, Ruiz-Ortega et al. 2001, Ruiz-Ortega, Lorenzo et al. 2001). Previously, angiotensin II was defined as a regulatory hormone that monitors blood pressure (Ruiz-Ortega, Lorenzo et al. 2001). Nowadays, it is accepted that angiotensin II could stimulate the cells regulating the expression of growth factors, cytokines, and adhesion molecules, which are engaged in cell apoptosis, inflammation and fibrosis (Egido 1996, Mezzano, Ruiz-Ortega et al. 2001, Ruiz-Ortega, Lorenzo et al. 2001). Moreover, RAS was demonstrated to have vital roles in activation of the cardiac fibroblasts, thus, it could be a suitable target for therapy (Reilly 2015). Unlike many

antihypertensive classes of drugs, such as β and calcium channel blockers, which did not show consistent positive results in humans, RAS inhibitors, such as ACE inhibitors, aldosterone antagonists and angiotensin II receptor blockers have been reported to have positive results in the treatment of cardiac fibrosis in both animals and humans (Roubille, Busseuil et al. 2014, Reilly 2015).

Fibrotic hearts express elevated levels of angiotensin II, which is an oligopeptide that causes vasoconstriction and increased blood pressure. Angiotensin II receptors (which are widely expressed in cardiac fibroblasts) have regulatory roles in the ECM production, adhesion and proliferation (Villarreal, Kim et al. 1993, Crabos, Roth et al. 1994). Moreover, lisinopril (ACE inhibitor) repressed myocardial fibrosis significantly (Brilla, Funck et al. 2000). Interestingly, cardiac fibroblasts express RAS components, and RAS signalling in these cells facilitates cell proliferation and migration. Hence, the effect of RAS blockers to treat cardiac fibrosis could be due to their anti-proliferative effects (Pinter and Jain 2017).

Furthermore, inhibition of the angiotensin I receptors in rat and mouse cell lines is anti-fibrotic by indirect activation of angiotensin II receptors and programmed cell death (Yamada, Horiuchi et al. 1996).

In contrast, angiotensin II heightens the activity of MAPK in human cardiac fibroblasts increasing the mRNA level of TGF-β and inhibiting the degradation of the ECM. All of which would further increase cardiac fibrosis (Kawano, Do et al. 2000). Moreover, clinical trials have shown that RAS inhibitors can decrease cardiac fibrosis in humans, although the study populations were rather small (Fang, Murphy et al. 2017).

1.9.2 TGF-β inhibitors

As mentioned before, TGF-β is a key cytokine with pro-fibrotic effects that regulates cardiac fibrosis, implying that inhibiting its action can have anti-fibrotic effects. TGF-β provokes fibrosis by retarding the degradation rate and heightening the production of ECM components (Edgley, Krum et al. 2012). TGF-β can be activated by a variety of stimuli such as: angiotensin II, MMP2 and MMP9. Interestingly, levels of MMP2 and TGF-β are increased in the cardiac tissues of human and animal heart failure. Moreover, TGF-β stimulates the expression of fibrillar collagen and fibronectin by cardiac fibroblasts (Edgley, Krum et al. 2012). Hence, inhibition of this cytokine is demonstrated to be a potential target for cardiac fibrosis (Reilly 2015). However, mice which received an anti-TGF-β antibody had a significant higher mortality rate after MI compared with the control group. Moreover, inhibition of TGF-β induced left ventricular remodelling (dilatation) after

MI. Although the production of collagen in mice treated with the TGF-β inhibitor was reduced, the expression of TGF pro-inflammatory cytokines were also increased (Frantz, Hu et al. 2008). Even though TGF-β promotes cardiac fibrosis, it also inhibits inflammation, which is essential for tissue repair. Hence, broad targeting and inhibiting TGF-β might not be a viable approach (Fang, Murphy et al. 2017). TGF-β inhibiting agents, such as pirfenidone and tranilast have been available clinically to affect the downstream targets of TGF-β, but both have adverse effects, such as liver dysfunction and liver failure (Fang, Murphy et al. 2017, Verma, Kumar et al. 2018).

1.9.3 Histone deacetylase inhibitors

One of the important groups of enzymes that play a crucial role in regulating and monitoring gene expression throughout are known as histone deacetylases (HDACs). The link between HDACs and cardiac fibrosis was first suggested by the discovery that these enzymes interact with members of the myocyte enhancer factor-2 (MEF2) transcription factor family (Konno, Chen et al. 2010). MEF2 regulates several genes that are involved cardiac hypertrophy. Konno and co-workers showed that levels of phosphorylated class II HDACs that activate MEF2 were increased in fibrotic rat hearts. It was also suggested that there is a link between MEF2-dependent gene activation and fibrosis (Konno, Chen et al. 2010).

Another investigation in mice demonstrated that silencing of the gene encoding HDAC9 heightens the activity of MEF2 (Zhang, McKinsey et al. 2002). In addition, mouse knockouts for HDAC5/9 develop exaggerated cardiac fibrosis by aging (Zhang, McKinsey et al. 2002, Chang, McKinsey et al. 2004). In contrast, overexpression of HDACs 4, 5 and 9 in cultured rat cardiomyocytes decreased the incidence of cardiac hypertrophy (Zhang, McKinsey et al. 2002, Bush, Fielitz et al. 2004, Backs, Song et al. 2006).

A later study in mice showed that HDAC inhibitors reverse cardiac remodelling and reduce cardiac fibrosis independent of angiotensin II in cardiac hypertrophy (Liu, Levin et al. 2008).

Since there are currently no Food and Drug Administration approved treatments for cardiac fibrosis, the pre-clinical results that highlight the roles of HDAC inhibitors in cardiac fibrosis could have significant clinical implications (Schuetze, Stratton et al. 2017).

1.9.4 Adenosine secretion and adenosine$_{2B}$ receptor stimulation

Adenosine has antimitogenic effects on isolated rat cardiac fibroblasts through the adenosine-2B receptor (A2BR), the receptor subtype, which is responsible for the inhibitory actions of adenosine on foetal calf serum (FCS) stimulated proliferation, collagen production and protein synthesis (Dubey, Gillespie et al. 1997). Adenosine induces inhibitory effects on FCS-induced collagen and

total protein synthesis in rat left ventricular cardiac fibroblasts, implicating the potentially important role of adenosine against cardiac fibrosis (Dubey, Gillespie et al. 1998).

The antifibrotic role of A2BRs (via adenosine) was further confirmed when anti-sense oligonucleotide to the A2BR induced DNA synthesis, collagen synthesis and cell proliferation. This demonstrates the potential inhibitory characteristics of adenosine and A2BR in the regulation of cardiac remodelling correlated by the proliferation of cardiac fibroblasts (Dubey, Gillespie et al. 2001). In contrast, over expression of A2BR in cardiac fibroblasts resulted in a significant reduction of protein and collagen synthesis associated with upregulation in cAMP. Conversely, significant upregulation of protein and collagen synthesis were observed when A2BRs were under expressed (Chen, Epperson et al. 2004). Over and above the effects on collagen synthesis, A2BR attenuates the mRNA expression of profibrotic gene markers, such as collagen 1 and connective tissue growth factor (CTGF) in neonatal rat cardiac fibroblasts pre-treated by angiotensin I or TGF-β (Vecchio, Chuo et al. 2016).

Additionally, *in vivo* experiments showed that, genetic over expression of A2BR in mice is antifibrotic, attenuates cardiac remodelling and is cardiac protective against pressure-induced heart failure (Hamad, Zhu et al. 2012, Vecchio, White et al. 2017). Likewise, extracellular cAMP protects the heart from adrenergically induced cardiac fibrosis and remodelling, an effect which was opposed when A2BR were antagonised pharmacologically. Together, these observations imply antifibrotic roles for cAMP and A2BRs (Sassi, Ahles et al. 2014). Moreover, an investigation in rats demonstrated long-term administration of adenosine uptake inhibitor and a stable adenosine analogue post MI had antifibrotic effects on hearts and attenuated cardiac remodelling by inducing a downregulation on the mRNA levels of ANF and BNP genes, both of which are useful markers of cardiac hypertrophy. Additionally, pharmacological agents that upregulate intracellular adenosine levels or stimulate A2BRs can reduce the markers of cardiac fibrosis, such as downregulation in the mRNA levels of MMPs and reduction in the collagen volume (Wakeno, Minamino et al. 2006). On the other hand, the antifibrotic effects of A2BR agonists were inhibited in the presence of A2BR antagonist (MRS1754), but the antifibrotic effects remained unchanged when other ARs were selectively antagonised (Wakeno, Minamino et al. 2006).

Studies on human myocardium demonstrated that, there is a significant reduction in the gene expression of all A2A, A2Bs and A3A adenosine receptors during heart failure. It was also shown that, pharmacological increase of plasma adenosine levels would reduce the severity of chronic heart failure in patients (Asakura, Asanuma et al. 2007). Together these findings suggest a protective

role of adenosine and A2BRs against cardiac fibrosis. However, this salutary effect might not be present in patients with chronic heart failure due to decreased receptor expression (Asakura, Asanuma et al. 2007, Vecchio, White et al. 2017).

The second messenger downstream of adenosine receptors, cAMP, has also been shown to have important roles in the inhibition of the activity of cardiac fibroblasts (Swaney, Roth et al. 2005, Lu, Aroonsakool et al. 2013). Consistent with this, investigations in adult rat cardiac fibroblasts demonstrated that, the expression of SMA (a marker of cardiac fibroblasts) was increased after incubation with TGF-β and angiotensin II, but this was inhibited following forskolin (adenyl cyclase activator) and cAMP analogue stimulation. Moreover, treatment with forskolin blunted the collagen synthesis induced by TGF-β and angiotensin II and inhibited the transformation of cardiac fibroblasts to myofibroblasts. These results could demonstrate the important role of cAMP in inhibiting ECM production and could imply antifibrotic effects (Swaney, Roth et al. 2005).

In addition to direct effects mediated through cAMP, adenosine has been shown to have antifibrotic effects via inhibiting the secretion of noradrenaline, attenuating the metabolism of endothelin and decreasing the production of renin-angiotensin system (Kitakaze and Hori 2000).

Taking all these results together implies that actions of adenosine, especially via A2BRs, is beneficial in the treatment of cardiac fibrosis. However, it has been reported that adenosine and its action via A2BRs can also have deleterious effects (Borea, Gessi et al. 2017).

For example, formation of foam cells and development of atherosclerosis are among the main factors of many cardiovascular diseases (Borea, Gessi et al. 2017) and can lead eventually to heart failure. An investigation carried out by Gessi and colleagues demonstrated that, in hypoxic conditions, adenosine through the stimulation of ARs (specially A2BRs), induces hypoxia inducible factor-1α (HIF-1α) accumulation. This is important because HIF-1α induces the development of foam cells and intraplaque angiogenesis, all of which could lead to atherosclerotic plaques (Gessi, Fogli et al. 2010). In contrast, genetic modifications of A2BRs or their pharmacological inhibition retarded the effects of adenosine on HIF-1α accumulation (Gessi, Fogli et al. 2010). Therefore, it was concluded that, adenosine and its receptors (mainly adenosine A2B receptors) play crucial roles in the development of coronary disease (Gessi, Fogli et al. 2010).

Adenosine might also play detrimental roles in different chronic pulmonary inflammatory conditions, such as; chronic obstructive pulmonary disease (COPD) and asthma (Borea, Gessi et al. 2017). Recently, an investigation in mouse models of pulmonary fibrosis reported that, extracellular adenosine levels are increased with pulmonary fibrosis. Furthermore, potentiating extracellular

adenosine levels by blocking its reuptake exacerbated the pulmonary fibrosis in these models (Luo, Le et al. 2016). Adenosine promotes the proliferation of a specific type of macrophages that is shown to be associated with pulmonary fibrosis. In another investigation, mice lacking A2BRs demonstrated lower pulmonary fibrosis and had their lungs function improved. These results were followed by a reduction in the expression of CD206 macrophages and arginase-1 (markers for alternatively activated macrophages). The author suggested that, adenosine and A2BRs play crucial roles towards the progression of lung fibrosis by having effects on the activation of macrophages (Karmouty-Quintana, Philip et al. 2015).

Since adenosine stimulation and activation of A2BRs can express both stimulatory and inhibitory effects on cardiac fibrosis, further clinical investigations are required to design a related treatment for cardiac fibrosis.

1.10 Adenosine

Adenosine is a ubiquitous nucleoside that is produced from the breakdown of adenosine triphosphate (ATP) (Newby 1984). The antiarrhythmic and vasodilatory properties of adenosine were the first to be discovered. Since then, it became clear that besides its role as an intermediate in cellular metabolism on nucleotides, extracellular adenosine plays multiple important physiological roles in cardiovascular system (Drury and Szent-Gyorgyi 1929, Mubagwa, Mullane et al. 1996).

Most roles are homeostatic and salutary in nature (Mubagwa, Mullane et al. 1996) but exceptions have been noted. For example, adenosine directly increases the rate of myocardial blood flow and oxygen supply (Adair 2005), whereas the antiadrenergic effects of adenosine could reduce the supply of oxygen in those tissues (Dobson and Fenton 1997, Adair 2005). Therefore, adenosine may act as a positive and negative feedback signal to tightly control the levels of oxygen in the tissues within the normal range (Adair 2005). Moreover, the well characterised cardioprotective actions of adenosine have made it a focus for clinical trials for ischaemia reperfusion injury after MI (Vecchio, White et al. 2017). Adenosine also plays important roles in the regulation of cardiac remodelling and cardiac fibrosis (Vecchio, White et al. 2017).

1.10.1 Metabolism of adenosine

Unlike other hormones or neurotransmitters, adenosine can be produced by any cell type and act locally whenever it is needed. The short half-life of adenosine extracellularly (a few seconds) would indicate that, it would be able to diffuse several millimetres form its production site, implying its wide sphere of action (Bruns 1990).

The main site of adenosine production in the heart are cardiac vascular endothelium and cardiomyocytes. Adenosine can be metabolised by two main pathways: the production of adenosine from AMP via dephosphorylation and the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine (Mubagwa, Mullane et al. 1996, Adair 2005). Figure 2 shows the synthesis of adenosine.

1.10.1.1 Dephosphorylation of adenosine monophosphate

The main source of adenosine synthesis during hypoxia is by the dephosphorylation of intracellular adenosine monophosphate (5'-AMP), the concentration of which is dependent on adenosine triphosphate (ATP) and energy demand (Zimmer, Trendelenburg et al. 1973, Headrick, Peart et al. 2011). The intracellular production of adenosine under hypoxic/ischaemia conditions takes place by the dephosphorylation of 5'-AMP via cytosolic-5'-nuclotidase or via cd73/ecto-5'-nucleotidase extracellularly (Berne 1980, Bruns 1990, Adair 2005).

1.10.1.2 Hydrolysis of SAH

Adenosine can also be produced through the transmethylation pathway, where SAH is broken down to adenosine and homocysteine by hydrolysis, a reaction that is catalysed by an enzyme known as SAH-hydrolase (Mubagwa, Mullane et al. 1996). In guinea pig hearts the rate of adenosine production via the hydrolysis of SAH is almost equal to the rate of the adenosine synthesis from the heart in normoxia, but it does not increase when there is a demand for oxygen (Lloyd and Schrader 1993).

1.10.2 Adenosine signalling

Adenosine exerts its cardiovascular effects through the stimulation of adenosine receptors (ARs) family, which are G-protein coupled receptors (GPCRs) located on the cell surface membrane (Vecchio, White et al. 2017). This family consists of 4 class A receptors: A1, A2A, A2B and A3. Each one of these sub-members exerts a specific pharmacological action via coupling to intracellular G proteins. The A1 and A3ARs tend to activate $G_{i/o}$ proteins to inhibit the activity of the adenyl cyclase (AC) enzyme and hence cAMP production. In contrast, the A2A and A2BR sub-types preferentially activate Gs proteins to activate AC and increase the intracellular levels of cAMP (Fredholm, AP et al. 2001, Vecchio, White et al. 2017). Synchronous activation of different ARs could therefore have complementary or opposing signalling effects to regulate cardiac function (Chan and Cronstein 2010, Headrick, Peart et al. 2011, Vecchio, White et al. 2017).

1.10.3 Adenosine breakdown

Adenosine can be broken down via two ways. It can be deaminated to inosine by an enzyme known as adenosine deaminase. Moreover, it can also get re-phosphorylated (using ATP as a donor) into AMP by adenosine kinase. Furthermore, diffusion plays an important role in the equilibration of adenosine. The nucleoside transporter is capable of transporting adenosine down its concentration gradient by diffusion (Adair 2005). Figure 1.2 demonstrates the breakdown of adenosine.

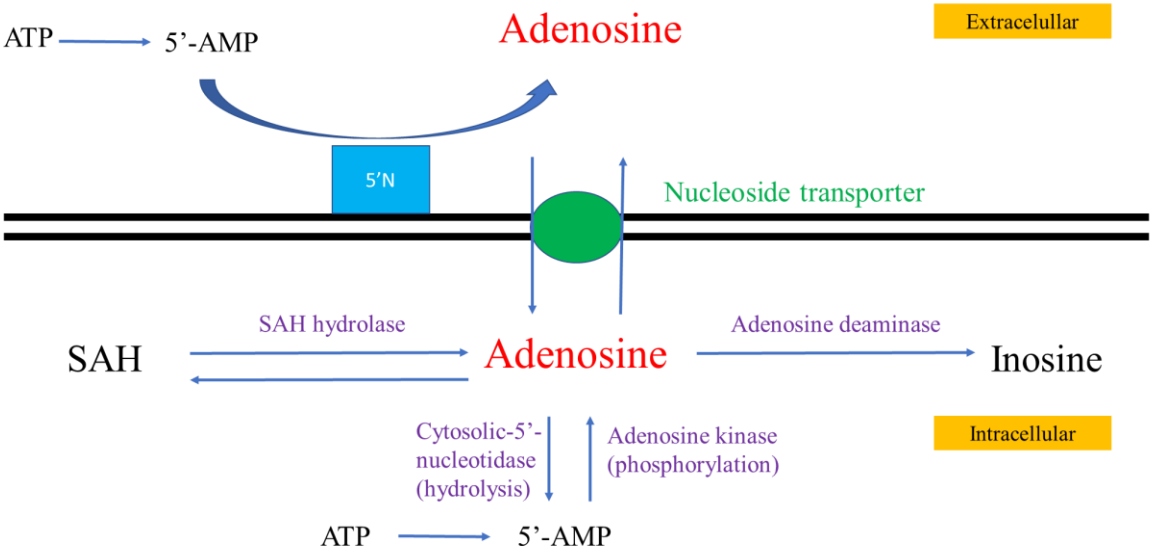


Figure 1.2: Adenosine synthesis and breakdown inside and outside the cell.

SAH: S-adenosylhomocysteine; 5'N: Ecto-5'-nucleotidase.

1.11 Mechanisms of cardiac fibroblasts proliferation

As mentioned earlier, one of the therapeutic ways to target cardiac fibrosis, is to inhibit their proliferation rate. Therefore, it is important to look at the steps involved in cell cycle and how cardiac fibroblasts divide.

1.11.1 Regulation of the cell cycle, including the importance of actin polymerisation

The proliferation of all diploid (double sets of chromosomes) cells, including cardiac fibroblasts, is controlled by progression through a mitotic cell cycle. Non-dividing (quiescent) cardiac fibroblasts are maintained in the G_0 phase, but they can be stimulated by suitable mitogenic signals to enter the first Gap period (G_1). It is this stage where the necessary factors for DNA replication are assembled, proteins and organelles are made and dividing cells spend most of their time (Vermeulen, Van Bockstaele et al. 2003). G_1 phase has a restriction point which divides it into 2 sub-phases. Prior to this point, the cell cycle is dependent on the growth factors, in other words, once the growth factors are removed, the cell cycle is forced back to G_0 phase. However, after this point, the cell cycle progression is independent of the growth factors (Blagosklonny and Pardee 2002). Synthetic (S) phase is the stage where DNA is replicated, which is then followed by a second Gap (G_2) phase. In G_2 cells are prepared for mitosis (M). M phase itself is sub-divided into 4 stages known as: prophase, metaphase, anaphase and telophase (Vermeulen, Van Bockstaele et al. 2003). To make sure that cell cycle progresses correctly, there are various check points. For example: any sort of damage to the DNA molecule would stimulate a replication arrest either in G_1 phase (when DNA repair occurs before replication) or in G_2 (to allow DNA repair prior to chromosome separation in M phase). Moreover, there are checkpoints in S phase to control DNA replication and M phase to monitor spindle assembly (Stark and Taylor 2004, Houtgraaf, Versmissen et al. 2006).

1.11.2 Positive regulation of the cell cycle

The transition of phases in the cell cycle are monitored by regulatory proteins known as cycle dependent kinases (CDKs). CDKs belong to the family of serine/threonine kinases. They are enzymes that phosphorylate their specific targets, in other words, addition of an extra phosphate group causes a change in the structure and shape of the target protein. The protein levels of CDKs remain stable during the cell cycle, however, the levels of their activating proteins (cyclins) can vary.

Up till now, 9 different CDKs (CDK1-9) along with 12 cyclins (A, B1-3, C, D1-3, E, F, G and H) have been discovered and identified in mammalian cells (Johnson and Walker 1999, Vermeulen, Van Bockstaele et al. 2003, Xie, Wu et al. 2005). 5 out of 9 of the CDKs are active during the cycle

of cell division. For example: CDK2, CDK4, and CDK6 during the G_1 phase, CDK2 during the S phase and CDK1 during the G_2 and M phases (Vermeulen, Van Bockstaele et al. 2003). In contrast, the crucial roles of the remaining CDKs have not been reported (Rickert, Seghezzi et al. 1996). Upon activation, CDKs require cyclins to form enzymes which phosphorylate specific proteins to control the progression of the cell cycle (Vermeulen, Van Bockstaele et al. 2003). Importantly CDK2/4/6 activation results in the inactivation by hyperphosphorylation of the retinoblastoma tumour suppressor protein (Rb) (Sherr 1995, Rubin 2013). When Rb is active (hypophosphorylated), it binds to a family of transcription factors, known as E2F and inhibits their activity, which maintains cells in the quiescence state. In contrast, once Rb is hyperphosphorylated by the activity of CDKs, they are no longer able to bind to E2F family of transcription factors, which can now be released, allowing the expression of the genes involved in S phase (Burke, Deshong et al. 2010).

In mammalian cells, there are important regulators that play key roles in the transition of G1 phase to S phase, such as all D-type cyclins and cyclin E. The three D-type cyclins bind to CDK4 and CDK6, whereas cyclin E makes a complex only with CDK2 (Sherr 1994). Cyclin E-CDK2 complexes further phosphorylate Rb gene at different sites, which would result in the synthesis of cyclin A. Cyclin A promotes passage via the restriction point to the S phase. Once S phase starts, cyclin E gets degraded and CDK2 makes a complex with cyclin A to phosphorylate the proteins involved in DNA replication. In the next stages of the cell cycle (late stages of G2 phase and early M phase), cyclin A complexes with CDK1 to promote mitosis. Also, the levels of cyclin B along with its CDK1 complex increase at the late stages of phase G2, which regulates cell division and mitosis (Vermeulen, Van Bockstaele et al. 2003).

1.11.3 Negative regulation of the cell cycle

In addition, CDK activities are also controlled by other mechanisms, such as phosphorylation on conserved threonine and tyrosine residues brought about by CDK activating kinase (CAK) (Vermeulen, Van Bockstaele et al. 2003). Phosphorylation on these residues introduces conformational changes which enhances the binding of the cyclins to CDKs (Jeffrey, Russo et al. 1995). In contrast, phosphorylation can also be a negative regulator and halt the cell cycle. For example, phosphorylation of CDK1 by Wee1 (at tyrosine-15) and Myt1 (at threonine-14) inactivates this kinase. On the other hand, dephosphorylation of these sites on CDK1 (which is brought about by an enzyme known as Cdc25) is necessary for its reactivation (Lew and Kornbluth 1996, Vermeulen, Van Bockstaele et al. 2003).

Furthermore, the activity of the CDKs can also be inhibited by cell cycle inhibitory proteins, known as CDK inhibitors (CKI). CKIs bind to CDKs alone or to the cyclin-CDK complexes and inhibit the CDK activity (Vermeulen, Van Bockstaele et al. 2003). CKIs are divided into two families: the INK4 and the Cip/Kip families (Sherr and Roberts 1995).

Briefly, the INK4 family is made up of 4 different proteins, namely p15, p16, p18 and p19. This family of CKIs specifically inhibits CDKs involved in the G1 phase of the cell cycle, such as: CDK4 and CDK6. Their mechanism of inhibition is by making stable complexes with CDKs and stopping them from making complexes with cyclin D (Carnero and Hannon 1998, Canepa, Scassa et al. 2007).

On the other hand, the Cip/Kip family is made up of three different proteins: p21, p27 and p57. Unlike the INK4 family, they have a broader range of specificity. In addition to the inhibition of the cyclin D-CDK4 and cyclin D-CDK6, they can also inhibit cyclin B-CDK1 and DNA synthesis (Hengst and Reed 1998).

1.11.4 Regulation of CDKs in the cell cycle of cardiac fibroblasts

Literature evidence suggested important roles of CDKs, cyclins and CKIs in the regulation of the cardiac fibroblast cell cycle (Qi, Liu et al. 2017, Valkov, King et al. 2019). Recently, an investigation in rats demonstrated that inhibition of CDK2 and cyclin E halted the progression of cell cycle in cardiac fibroblasts. In addition, increased expression of CKIs, such as p21 (in rat hearts) and p27 (in cultured cardiac fibroblasts) downregulated the interstitial cardiac fibrosis. In contrast, inhibiting the activities of CKIs by gene therapy induced DNA synthesis, suggesting the involvement of these CKIs in the inhibition of cardiac fibroblast proliferation (Qi, Liu et al. 2017).

Several cytokines and growth factors are implicated in the regulation of myocardial growth during developmental and pathologic conditions (Koudssi, Lopez et al. 1998). Interleukin-1β is a polypeptide secreted by different types of cells, such as smooth muscle cells, epithelial cells and fibroblasts (Dinarello 1994). The expression of this polypeptide by myocardial cells has been detected in several clinical circumstances characterised by inflammatory myocardial injuries. Cardiac fibroblasts are important sources of interleukin-1β, which upregulates the protein content of myocytes but downregulates the DNA synthesis in cardiac fibroblasts (Koudssi, Lopez et al. 1998). Investigations on isolated rat cardiac fibroblasts have shown that interleukin-1β inhibits the progression of the cell cycle by preventing entry into S phase and arresting them in the G1/S interface. Moreover, interleukin-1β also decreases the phosphorylation state of Rb in these cells, thereby maintaining them in the quiescence state. Also, it decreases the expression of CDK2, CDK4

and cyclins, such as: A, D2, D3 and E (Koudssi, Lopez et al. 1998). These findings demonstrate the roles of these proteins in the cell cycle of cardiac fibroblasts and emphasise the potential importance of targeting them for future treatments.

As mentioned previously, once cardiac fibroblasts are activated, they can migrate to different areas within the tissue. Consequently, it is important to delve deeper into the mechanism of cardiac fibroblast migration.

1.12 Mechanisms of cardiac fibroblasts migration

Cell migration plays essential roles in health and diseases states, such as embryogenesis, tissue development, immune response and wound healing (Lauffenburger and Horwitz 1996, Mitchison and Cramer 1996, Kole, Tseng et al. 2005). Directional motility consists of different steps: protrusion of lamellipodia (parts of the cellular cortex) at the leading edge; attachment (adhesion) at the leading edge; deadhesion of the rear (trailing edge); and movement of the cell (Kole, Tseng et al. 2005, Petrie, Gavara et al. 2012). Cell migration occurs by the assembly, disassembly and reformation of filament structures (also known as the actin cytoskeleton), that are regulated by members of Rho family of small GTPases, such as Rac, Cdc42 and RhoA (Nobes and Hall 1999). Genetic modifications have demonstrated that Rac is important for membrane protrusion and Cdc42 is necessary for the maintenance of cell polarity (Nobes and Hall 1999). RhoA, on the other hand, plays important roles in the regulation of actin dynamics, encouraging the contraction of acto-myosin by controlling myosin II and formins (Sixt 2012, Zhou and Zheng 2013).

Actin is among the most abundant proteins in eukaryotic cells; and actin filaments are the dominant structural components in lamellipodia. Actin filaments are structures made up of double helical polymers of globular subunits, with head to tail arrangements. The structure has two ends: the barbed end and the pointed end (Pollard and Borisy 2003). Polymerisation of actin at the leading edge leads to the formation of sheet like (lamellipodia) and rod like (filopodia) protrusion at the cell membrane (Nobes and Hall 1995). In lamellipodia, actin filaments cross together and form a meshwork-like network, while in filopodia, they are arranged into bundles of ropes (Lauffenburger and Horwitz 1996).

At the leading edge of the cell, polymerisation of actin filaments provides the cells with the necessary pushing force. This requires the addition of actin monomers (G-monomers) to the plus or barbed end of actin filaments via formins, such as: mDia1 and mDia2, which involves the interaction with G-actin-bound-profilin. In addition to this, a stronger force can either be achieved by the development of branched actin filaments or, by an upregulation in the density of actin filaments via

the help of an enzyme known as cofilin, which increases actin polymerisation by producing additional free barbed ends (Mogilner and Oster 1996). Cell migration is also dependent on contractile forces at the trailing edge of the cell. Behind the leading edge, actin filaments are arranged into bundles to form stress fibres that criss-cross the cell and associate with myosin II to enhance the movement of the rear end of the cell. Force production at the leading and the trailing edge of the cell is dependent on the adhesion with ECM (Webb, Parsons et al. 2002). These contacts are facilitated by integrin adhesions, shaped at the leading edge and matured into focal adhesions. The focal adhesions are structures where F-actin fibres are attached; and they finally get disassembled at the trailing edge of the cells. Due to multiple protein interactions present in focal adhesions, such as: PI3K; focal adhesion kinase (FAK); and MAPKs; focal adhesions can also act as signalling complexes (Wozniak, Modzelewska et al. 2004). Figure 1.3 summarise the steps involved in the cell migration.

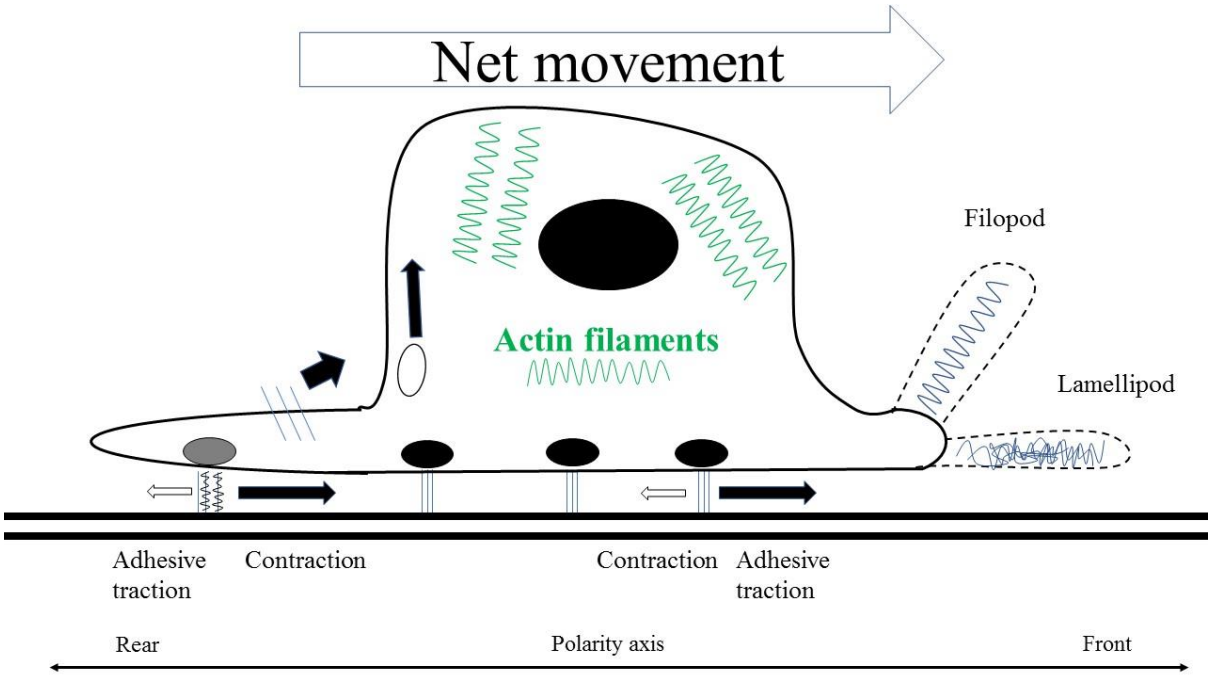


Figure 1.3: Steps involved in the migration of cardiac fibroblasts

There are distinctive steps involved in the migration of cells with mesenchymal origin. Once the direction of the movement of the cell is determined, actin polymerisation at the front of the cell forms membrane protrusions via two means: filopodia and lamellipodia. Following the extension of the cell membrane, it connects its leading edge to the surface (ECM) over which it is moving by the formation of integrin-based complexes. Eventually, the resulting focal adhesions get matured and F-actin fibres are attached. Contractile forces are applied to the focal connections via myosin II motors to enhance the migration of the cells. As the cell migrates, it detaches from the ECM at the cell body and the rear, inducing cell contraction and facilitating the movement of the cell. Finally, the cell moves forward via the help of contractile forces (Lauffenburger and Horwitz 1996).

1.12.1 Regulation of migration by Rho-GTPases

Rho-GTPases regulate the coordination of cell migration and actin polymerisation. Guanine nucleotide exchange factors (GEF proteins), exchange GDP (inactive form) to GTP (active form) (Jaffe and Hall 2005). In humans, the Rho family consists of 20 different members. Among the members, RhoA, RhoB, Rac1, Rac2 and Cdc42 have been among the most widely investigated for their roles and effects on cell migration (Ridley 2001). Rho-GTPases can get inactivated via two ways: guanine nucleotide (GDP) dissociation inhibitors (GDIs) and GTPase activating proteins (GAPs). GDI binds to the inactive form of RhoA and prevents the exchange of GDP for GTP; and also inhibits the interaction with cell membrane (Carpenter, Tolias et al. 1999, Hansen and Nelson 2001). On the other hand, GAPs can catalyse the dephosphorylation of GTP to GDP (Cherfils and Zeghouf 2013). Among the members of the Rho-GTPases, RhoA, Rac1 and Cdc42 can regulate the activity of proteins modulating actin polymerisation in order to control the formation of F-actin fibres, lamellipodia and filopodia (Nobes and Hall 1995, Tapon and Hall 1997, Hall 1998). As noted above, actin polymerisation at the leading edge of cardiac fibroblasts (eukaryotic cells) arises from the well-coordinated activities of different nucleating proteins, such as: Formins (mDia1 and mDia2) and Arp2/3 (Swaney and Li 2016).

Rac1, Cdc42 and RhoA regulate the assembly of cytoskeleton in different ways (Hall 2005, Sit and Manser 2011). Rac1 activates Arp2/3 through the activation of WASP family verprolin-homologous protein (WAVE) and it regulates the formation of lamellipodia (Takenawa and Miki 2001, Hall 2005). Like Rac1, Cdc42 also activates Arp2/3 via Wiskott-Aldrich syndrome protein (WASP) and N-WASP (Higgs and Pollard 2000). In addition, Cdc42 also activates mDia2, resulting in the regulation of filopodia (Hall 2005). On the other hand, mDia1 gets activated by RhoA to enhance linear actin polymerisation and regulate the formation of stress fibres (Hall 2005, Lammers, Meyer et al. 2008). Moreover, the activation of RhoA by Rac1 results in the development of new sites of adhesion and the formation of contractile filament assembly with an advancing lamellipodium (Nobes and Hall 1995).

Importantly, the two GTPases, Rac1 and RhoA have antagonistic effects on actin-myosin contraction (Kirfel, Rigort et al. 2004, Martin, Ouellette et al. 2016). RhoA activity results in the activation of a kinase known as Rho-kinase (ROCK), which is induced by the binding of Rho to the Rho-binding domain on the COOH terminal (Phrommintikul, Tran et al. 2008). ROCK has two isoforms: ROCK1 and ROCK2 (Zhang, Bo et al. 2006). Upon activation, ROCK phosphorylates and inactivates an enzyme known as myosin light chain phosphatase (MLCP), which regulates myosin light chain (MLC) (Wilkinson, Paterson et al. 2005). Phosphorylated MLC provides the

necessary contractility force and induces RhoA-mediated reformation of actin stress fibres, an important mechanism in cell migration that is depended on ROCK (Totsukawa, Yamakita et al. 2000). One of the other protein targets of ROCK is LIM-kinase (LIMK), which contributes to the actin polymerisation by inhibiting cofilin and stabilising F-actin fibres (Amano, Nakayama et al. 2010). Equally, MLC kinase can get phosphorylated and inactivated by the activation of p21-activated kinase (PAK1) via Rac1. This results in the downregulation of contractile forces and upregulation of cell migration (Martin, Bollag et al. 1995). Cell polarity is a necessary process for cell migration. Polarity is regulated not only via the contractile forces mediated by the arrangement of actin filaments and deadhesion at the rear end of the cell, but also via proper orientation positioning microtubule organising centre (MTOC) at the leading edge of the cell (Gomes, Jani et al. 2005).

Rho-GTPases were initially recognised as a molecular switches, which associate integrin receptors on the cell membrane to rearrangements of the actin cytoskeleton and have emerged as crucial targets for cardiovascular disease (Budzyn, Marley et al. 2006). As cells change the arrangements of their actin cytoskeleton in response to stimuli, such as growth factors, or during mitosis and cell migration, they alter the localization of their intracellular proteins by regulating Rho-GTPases (Rajagopalan, Kurz et al. 1996). Rho-GTPases can also influence cellular processes, such as membrane trafficking, mRNA stability and gene transcription (Laufs and Liao 2000). Elevated RhoA and Rac1 activities induce cardiac hypertrophy atrial fibrillation (Clerk and Sugden 2000, Adam, Frost et al. 2007). In contrast, inhibiting RhoA either by gene expression or using selective inhibitor for RhoA (by *Clostridium botulitum* C3 coenzymes) retards cardiac remodelling effects caused by angiotensin II (Aikawa, Komuro et al. 1999).

Like the Rho-GTPases, the Rho-ROCK pathway plays important roles in regulating different cellular functions, such as organisation of F-actin filaments, cell adhesion, proliferation and migration, which are all involved in the pathogenesis of cardiac fibrosis (Shimokawa and Rashid 2007, Haudek, Gupta et al. 2009). Consistent with this, studies in mice demonstrated that the mRNA and protein levels of ROCK1 were increased in fibrotic hearts induced by pressure overload (aortic constriction). Conversely, ROCK1 knockout mice demonstrated reduced non-adaptive cardiac fibrosis and lower differentiation of cells into cardiac myofibroblasts (Zhang, Bo et al. 2006). Moreover, ROCK haplo-insufficient mice had lower perivascular fibrosis induced by pressure overload and MI (Rikitake, Oyama et al. 2005). One way that ROCK promotes fibrosis is by promoting cardiac fibroblast proliferation. ROCK promotes proliferation via its actions on MKL1/2 (SRF cofactors, which will be discussed later in detail). ROCK stimulates nuclear translocation of

MKL1/2 and thereby promotes the transcription of SRF/MKLs target genes (Fan, Sebe et al. 2007, Elberg, Chen et al. 2008). Hence, Rho-GTPases, and at least in some cases ROCK, therefore have important roles in the regulation of cardiac fibroblasts and dysregulation of the Rho-ROCK-actin pathway can be a crucial mediator of cardiac fibrosis (Rikitake, Oyama et al. 2005). However, the role of ROCK in regulating cardiac fibroblast migration is less clear. Investigations in Gerbil fibroblasts demonstrated that these cells migrated faster upon the inhibition of ROCK by a specific ROCK inhibitor, Y27632 (Totsukawa, Wu et al. 2004).

1.13 Transcriptional regulation of cardiac fibroblasts

Following any cardiac injury, the sum of all the transcriptional modifications of cardiac fibroblasts could lead to pathological changes in their behaviour (Lighthouse and Small 2016). The transcriptional regulations of cardiac fibroblasts could be affected by different pathological stimuli, such as stimulation of the receptors on the cell surface membrane or/and modifications in their mechanical tensions (Lighthouse and Small 2016). Mechanisms which target the phenotypic plasticity of cardiac fibroblasts are discussed below.

1.13.1 SRF/MKL/RhoA

Serum response factor (SRF) is a conserved transcription factor that is important for life. It was first discovered in investigations on proliferative responses to serum (Esnault, Stewart et al. 2014). However, the binding site of SRF (CArG) is also important for myocardial (and other muscle cell) expression of differentiation genes, such as the sodium calcium exchanger and cardiac alpha actin (Cheng, Hagen et al. 1999, Latinkic, Cooper et al. 2002). The selection of SRF target genes and the scale of their transcriptional activation depend on the interactions with co-factors (Norman, Runswick et al. 1988). For example, the expression of genes encoding for SMC contractile proteins, which is also mediated by the CArG element, is effectively regulated by the interaction of SRF and members of the family of transcription cofactors related to myocardin (Miano 2003).

This family of cofactors consists of different members, such as myocardin, megakaryoblastic leukemia1 (MKL1) and MKL2 (Pipes, Creemers et al. 2006, Gau, Veon et al. 2017). Myocardin functions primarily in SMC and cardiomyocytes. It mediates the transcription of SMC differentiation genes and plays important roles in heart development (Wang, Chang et al. 2001, Du, Ip et al. 2003). On the other hand, MKL1 and MKL2 are widely expressed and can be translocated from nucleus to cytoplasm by Rho activation (Miralles, Posern et al. 2003). The shuttling mechanism between nucleus and cytoplasm is defined by their RPEL domain i.e. amino acids arginine, proline, glutamic acid and leucine (Guettler, Vartiainen et al. 2008). In fibroblasts, under

basal conditions, the regulation of MKL localisation and its interaction with SRF is controlled by the interaction of its RPEL domain with monomeric G-actin.

In other words, when F-actin is polymerised, the amount of monomeric G-actin is reduced, allowing the translocation of MKL into the nucleus where it binds to SRF and increase the transcription of SRF target genes (e.g. CCN1 and CTGF), resulting in more proliferation and migration (Cen, Selvaraj et al. 2003, Selvaraj and Prywes 2004). In contrast, depolymerisation of F-actin filaments causes an increase in the pool of G-actin, meaning more interaction and lesser translocation of MKL1/2 into the nucleus, inducing lesser transcription of SRF target genes (Guettler, Vartiainen et al. 2008). It implies that MKL1/2 regulate the phenotypic responses of fibroblasts by linking modifications in the actin filaments to the regulation of SRF target genes (Guettler, Vartiainen et al. 2008). Figure 1.4 shows the domain structures of myocardin, MKL1 and MKL2.

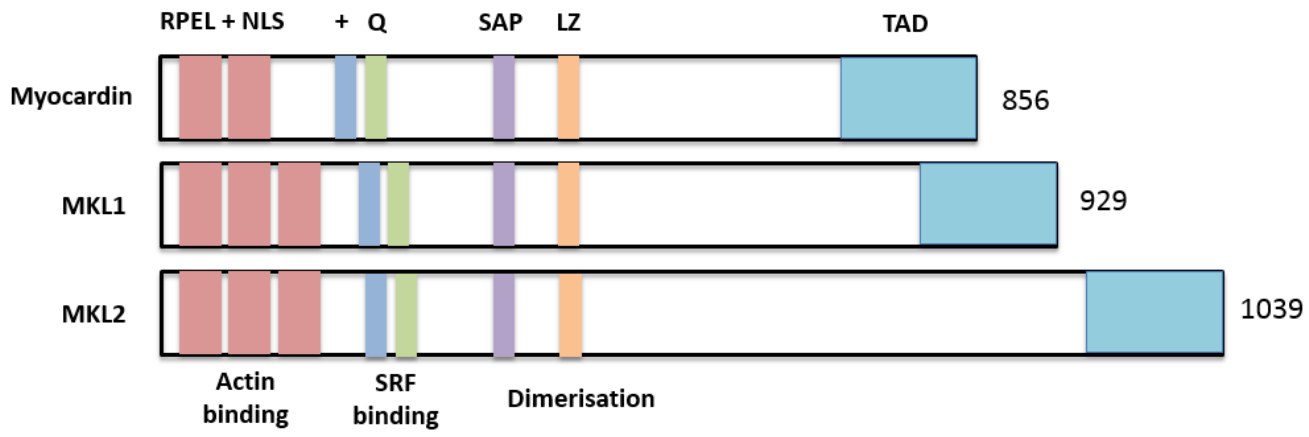


Figure 1.4: Structures of myocardin, MKL1 and MKL2 with their different domains

Myocardin-related transcription factors (MRTFs) share conserved domains, namely RPEL, NLS, basic, glutamine rich (Q), scaffold-attachment factor A/B, Acinus, PIAS (SAP), leucine zipper (LZ) and transcription activation (TAD) domains. The RPEL domains regulate actin binding in MKL1 and MKL2. This domain also contains an NLS sequence, meaning that actin binding can alter the subcellular localisation of MKL1 and MKL2. In contrast, in myocardin these domains have diverged to the point that myocardin does not associate with G-actin. The basic, glutamine rich and transcription activation domains are required for SRF-binding. The LZ domain regulates homo- and heterodimerisation with other MRTF family members. The numbers to the right indicate the number of amino acids (aa) in each protein (Guettler, Vartiainen et al. 2008)

1.13.2 The function of SRF/MKLs/RhoA

Chromatin immunoprecipitation assays and microarray studies documented groups of SRF-target early growth response genes, including c-fos and Egr1, which are co-regulated by MKL1 in fibroblasts (Selvaraj and Prywes 2004). These genes regulate proliferation in the cell cycle by promoting the transition of G0 to G1. Fibroblasts gene ontology analysis of the SRF/MKL target genes also uncovered additional genes associated with dynamics of actin filaments, proliferation and migration (Esnault, Stewart et al. 2014).

In the light of the roles of MKL1/2 as downstream mediators of ROCK signalling, other recent investigations also uncovered the importance of regulating MKL1/2 in fibroblast activation. For example, MKL1 knockout mice showed lower cardiac fibrosis post MI and displayed lower fibrosis in response to angiotensin II (a mediator of post-MI fibrosis). It was implied that the protective effects of MKL1 deletion were linked with reduction in expression of genes associated with fibrosis, such as collagen 1α2 (Small, Thatcher et al. 2010). Moreover, pulmonary fibrosis caused by bleomycin was ameliorated in MKL1 knockout mice via reduced myofibroblast proliferation (Bernau, Ngam et al. 2015). MKL1 may therefore play important roles in the regulation of myofibroblast phenotype (Lighthouse and Small 2016). In contrast, global genetic deletion of MKL2 or SRF in mice resulted in structural cardiac abnormalities and induced embryonic lethality (Arsenian, Weinhold et al. 1998, Mokalled, Carroll et al. 2015).

Recent investigations have identified MKL1/2 as potential therapeutic targets to regulate fibroblast responses (Lighthouse and Small 2016). Studies in human dermal fibroblasts demonstrated that MKL1 mediates fibroblast contractility, whereas MKL1 deficient fibroblasts failed to express genes encoding for SMC contractile proteins i.e. impaired myofibroblast transformation. The authors also identified, N-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide (ISX) as a stimulator of fibroblast activation in a MKL1 dependent manner. ISX induces the differentiation of human fibroblasts to myofibroblasts (which is dependent on MKL1/SRF) *in vitro* and it also accelerates cutaneous wound healing *in vivo* (Velasquez, Sutherland et al. 2013). On the other hand, MKL1/2 can be pharmacologically inhibited, as their RPEL domains are the molecular targets for CCG-1423, an inhibitor of Rho signalling (Hayashi, Watanabe et al. 2014). Pharmacological inhibition of MKL1/2 can suppress lung, colonic and dermal fibrosis in vivo (Evelyn, Bell et al. 2010, Haak, Tsou et al. 2014, Johnson, Rodansky et al. 2014).

1.13.3 Hippo pathway/YAP/TAZ/TEAD

The hippo pathway, is the most recently discovered family of signalling pathways that controls the size of the organs by mediating cell differentiation, proliferation and migration (Del Re 2018). It is an evolutionary conserved Serine/Threonine signalling pathway first discovered in fruit flies (Harvey, Pfleger et al. 2003). The core component of this signalling pathway are mammalian sterile 20-like kinases (MST1 and MST2), the regulatory protein salvador (SAV1), large tumour suppressor kinases (LATS1 and LATS2) and Mps one binder kinase activator-like 1A (MOB1A and MOB1B) (Rognoni and Walko 2019). Upon activation, MST1 and MST2 attach to SAV1 to produce an active enzyme that activates LATS1 and LATS2 kinases via phosphorylation at Thr1079 and Thr1041 respectively (Chan, Nousiainen et al. 2005). MST1 and MST2 can also phosphorylate MOB1A/B (which are regulatory subunits of LATS1 and LATS2) at Thr35 and Thr12 respectively (Praskova, Xia et al. 2008). In addition, phosphorylation at MOB1A and MOB1B leads to the auto-phosphorylation of LATS1 and LATS2 (Boopathy and Hong 2019). In order for LATS kinases to be active, both phosphorylation of MST1/2 and auto-phosphorylation of LATS1/2 kinases are required (Galan and Avruch 2016).

Activated LATS1/2-MOB1A/B can then phosphorylate the two major cofactors of transcriptional enhancer factor domain (TEAD1-4), known as yes associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ). YAP has two tryptophan domains (WW1 and WW2) and can be phosphorylated at 5 different serine/threonine sites: Ser61, 109, 127, 164 and 381. On the other hand, TAZ has one WW domain and can be phosphorylated at 4 serine/threonine sites: Ser66, 89, 117 and 311 (Zhao, Wei et al. 2007, Hao, Chun et al. 2008, Lei, Zhang et al. 2008). Once YAP and TAZ are phosphorylated, they either get recruited to14-3-3 proteins leading to cytoplasmic localisation (Zhao, Wei et al. 2007, Lei, Zhang et al. 2008) or get ubiquitinated leading to their proteolytic degradation (Zhao, Li et al. 2010). Any mutation on the serine residues of YAP and TAZ (mentioned above) makes these TEAD cofactors unresponsive to the hippo pathway (Piccolo, Dupont et al. 2014). Figure 1.5 demonstrates the structure of YAP and TAZ.

In contrast, when LATS1/2 kinases are not active, YAP/TAZ are no longer phosphorylated and are not bound to 14-3-3 proteins. They get translocated into the nucleus, where they can bind to the TEAD transcription factors to regulate the transcription of TEAD target genes, such as: connective tissue growth factor (CTGF); cysteine-rich angiogenic inducer 61 (CYR61) also known as cellular communication network factor (CCN1); plasminogen activator inhibitor 1 (PAI1); zyxin (ZYX); and others to regulate cell survival, growth, proliferation and migration (Chen, Chen et al. 2001, Lei, Zhang et al. 2008, Pobbati, Chan et al. 2012, Nardone, Oliver-De La Cruz et al. 2017, Smith,

Sessions et al. 2019). Figure 1.6 demonstrates the hippo signalling pathway. Studies in mice demonstrated that active YAP (a mutation on YAP that increases its nuclear localisation and activity) resulted in an increase in liver size and proliferation rate (Camargo, Gokhale et al. 2007). Furthermore, YAP activation in mice induced tumorigenesis, which might be due to the increase in the transcription rate of CCN1 and CTGF genes (Dong, Feldmann et al. 2007, Zhao, Ye et al. 2008). Overall, loss of any of the main components of the hippo pathway, such as MSTs, SAV1, MOB1A/B and LATS1/2 kinases, should turn off this signalling pathway. This increases the transcription rate of the YAP/TAZ/TEAD target genes that are required for cell growth, proliferation and migration (Boopathy and Hong 2019). However, an important thing to discuss is how YAP/TAZ/TEAD are regulated in the hippo pathway.

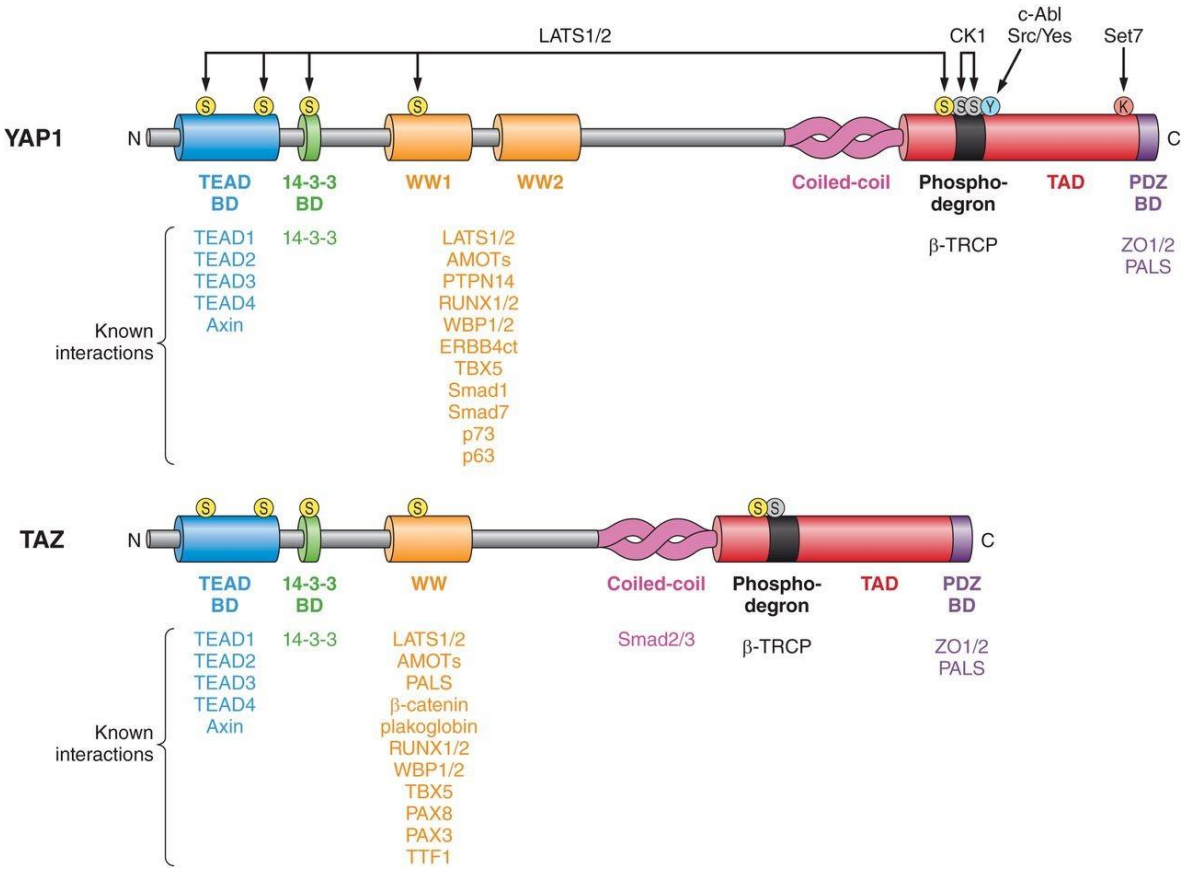


Figure 1.5: Schematic representation of YAP and TAZ proteins with their domains and posttranslational modification sites

The phosphorylation sites (serine) targeted by different kinases are demonstrated with circles in different colours. BD: Binding domain; TAD: Transcriptional activation domain; PDZ BD: Small COOH-terminal domain that is capable of interaction with proteins having the PDZ domains in their structures (Piccolo, Dupont et al. 2014).

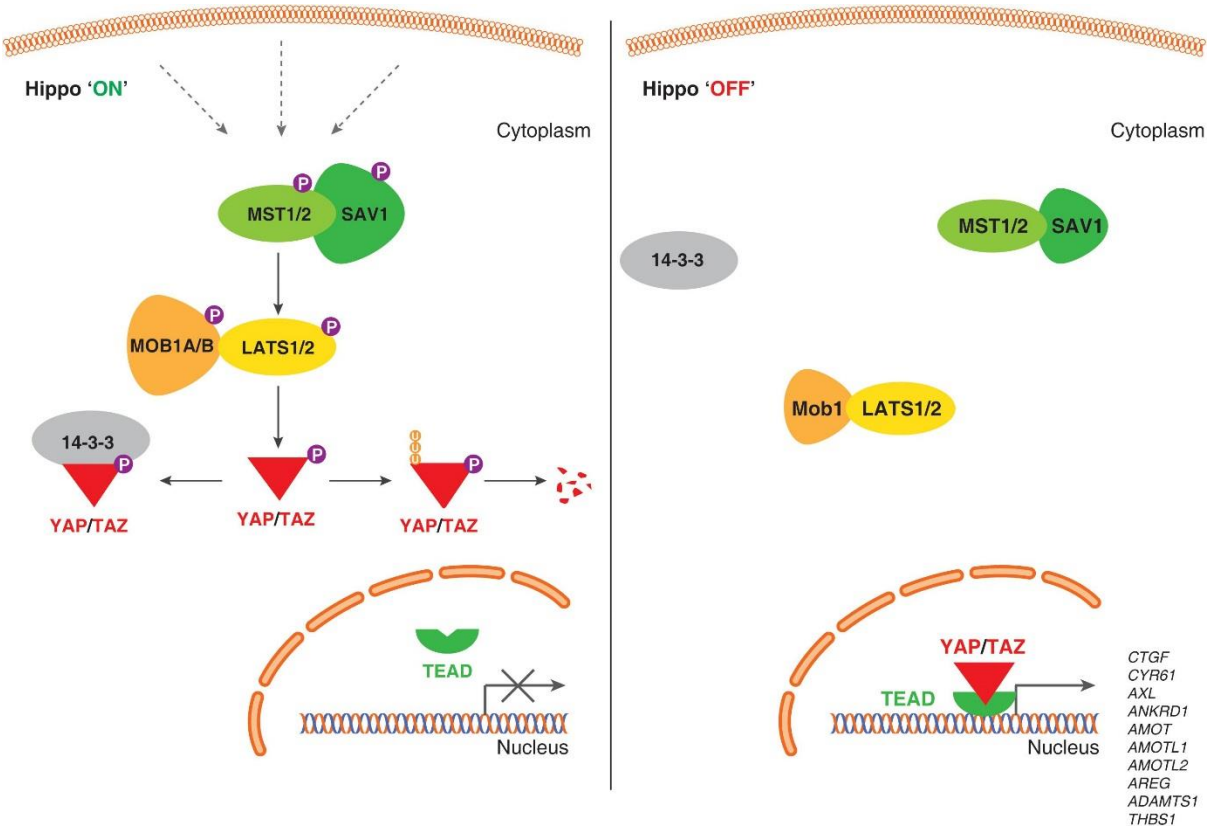


Figure 1.6: Schematic diagram representing the Hippo signalling pathway in mammalian cells

Left, When the signalling pathway is active, upstream signals mediate the phosphorylation of MST1/2, LATS1/2, YAP and TAZ proteins. Upon YAP and TAZ phosphorylation, they either get bound to 14-3-3 proteins and localised in cytoplasm or get proteolytically degraded. **Right**, when the signalling pathway is off, YAP and TAZ are not phosphorylated. They get translocated into the nucleus, where they bind to the TEAD element to mediate the transcription of TEAD target genes (Boopathy and Hong 2019).

1.13.4 Mechanical signals regulating YAP/TAZ/TEAD

Like other cell types, cardiac fibroblasts are subjected to different mechanical forces, such as stress and strain (Boopathy and Hong 2019). These mechanical inputs are produced by the stiffness of the ECM and by the pulling force generated by the neighbouring cells (Piccolo, Dupont et al. 2014). The mechanics of the cells and the organisation of their actin cytoskeleton are central inputs that cells use control their proliferation and migration in response to growth factors (Piccolo, Dupont et al. 2014). Importantly, the localisation (activity) of YAP/TAZ in response to these mechanical inputs is dependent on the status of cytoskeleton and the activity of Rho-GTPases (Zhao, Wei et al. 2007, Piccolo, Dupont et al. 2014, Boopathy and Hong 2019).

When a cell is stretched over a matrix, the formation of F-actin filaments induces the nuclear localisation and activation of YAP/TAZ, encouraging cell proliferation and migration (Dupont, Morsut et al. 2011). In contrast, when the morphology of the cell is changed into a rod shape, the F-actin filament are disturbed, resulting in the cytoplasmic translocation of YAP/TAZ, inhibiting their transcriptional activation capacity and retarding cell proliferation and migration (Rajkumar, Qureshi et al. 2017).

1.13.5 The function of YAP/TAZ/TEAD in the heart

YAP/TAZ/TEAD play crucial roles in the development of heart. Studies introducing mutation in TEAD1 gene in mice showed cardiac abnormalities, such as thinning of the cardiac ventricular walls and oedema. Also, it was suggested that TEAD1 plays central roles in the maturation stages of cardiogenesis (Chen, Friedrich et al. 1994)

Several lines of evidence suggested that YAP/TAZ-TEAD complex plays crucial roles in promoting fibrosis. For example, YAP activation has been shown to enhance cardiac fibroblast activation and in matrix remodelling of dilated cardiomyopathy (Jin, Zhu et al. 2018). Also, investigations in rodents demonstrated that conditional deletion of LATS1 and LATS2 in cardiac fibroblasts, which normally repress YAP and TAZ activity, induced cardiac fibrosis in mice (Xiao, Hill et al. 2019). In Zebrafish models of cardiac injury, hearts with YAP mutants demonstrated lower collagen synthesis at seven days post-cryoinjury, implying important roles of YAP in scar formation during heart regeneration (Flinn, Jeffery et al. 2019). Moreover, YAP knockout in primary rat cardiac cells revealed a fibroblast-specific role for YAP in regulating pro-inflammatory cytokines and chemokines, implying that YAP-TEAD-mediated gene expression may also have crucial roles in promoting inflammation, which is also an important factor prompting the fibrotic response (Flinn, Jeffery et al. 2019).

However, the role of YAP-TEAD in cardiac fibrosis is controversial. Some studies suggest an anti-fibrotic role of YAP. For example, over expression of YAP in the hearts of mice reduces scarring and fibrosis after MI. Moreover, quantification of fibrotic area displayed higher cardiac fibrosis in hearts of the control mice compared to YAP overexpressed mice. Furthermore, hearts of wildtype mice injured at postnatal day 2, were fully healed within 26 days, displaying minimal scarring. On the other hand, mice with YAP knockout hearts, showed a deficiency of healthy myocardial tissues along with large fibrotic area (Xin, Kim et al. 2013). Also, homozygous deletion of YAP elicits myocardial fibrosis compared to a sham group and a heterozygous YAP deleted group, leading to ventricular remodelling. It is not clear why these studies suggest opposite roles of YAP in regulating cardiac fibrosis. However, this may reflect different functions of YAP in cardiac fibroblasts and cardiomyocytes. For example, heterozygous YAP inactivated mice hearts displayed enhanced myocyte apoptosis (Del Re, Yang et al. 2013), which may exacerbate myocardial injury and indirectly promote fibrosis.

1.14 cAMP: synthesis, degradation and signalling

1.14.1 cAMP synthesis

The 3'-5' cyclic nucleotide, cAMP, is derived from ATP catalysed by adenylate cyclase (AC). The structure of an AC comprises two groups of transmembrane helices. This enzyme belongs to a larger family consisting of six different classes. In mammals, cAMP is produced by the third group of this family (class III ACs), consisting of ten members, nine of which belong to the transmembrane AC (tmAC) sub-family and one belongs to soluble AC (sAC). Cardiomyocytes do not express all the members. In fact, the expression of tmACs 1, 5, 6, 8 and sAC have been confirmed and most of them modulate inotropic and chronotropic outputs from β-adrenergic receptors. The activity of tmAC is modulated by neurotransmitters and hormones, and the mechanisms responsible for the activation and inhibition of tmACs are similar between all the nine members. Stimulation and inhibition are controlled via G_s and G_i proteins respectively, although there are some differences between them in their regulation by different PKAs. The other source of ACs, sAC, does not have any transmembrane domain and exists throughout the cell (i.e. mitochondria and nucleus). This cAMP source has the ability to produce cAMP molecules in the vicinity of the specific targets (Willoughby and Cooper 2007, Boularan and Gales 2015). Figure 1.7 demonstrates the structure of an AC.

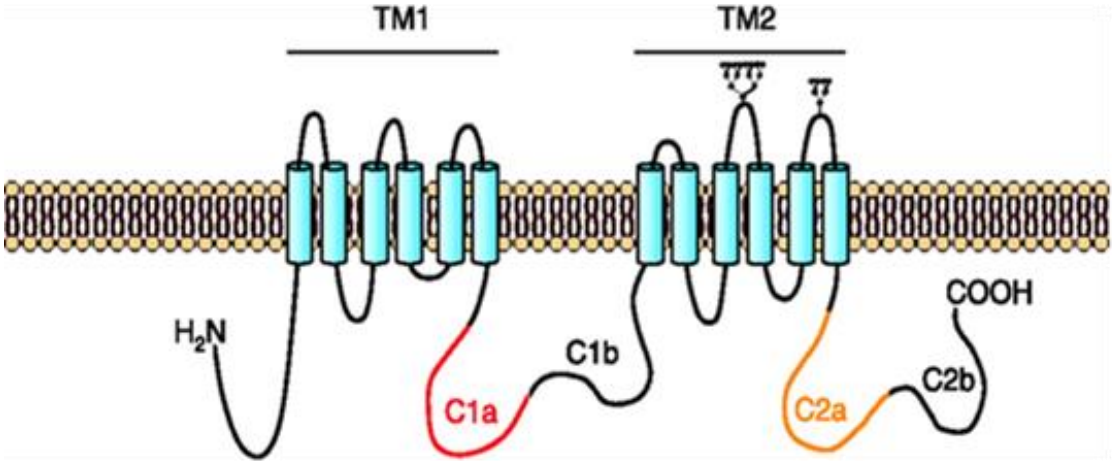


Figure 1.7: Structure of an AC

The structures of ACs are made up of two transmembrane regions comprising six helices each (represented by TM1 and TM2) and to cytoplasmic (C1 and C2) domains. NH_2: N-terminal; COOH: C-terminal (Omori and Kotera 2007, Willoughby and Cooper 2007).

1.14.2 cAMP degradation

The intracellular concentration of cAMP is maintained and controlled via two mechanisms: hydrolysis by phosphodiesterase (PDE); and efflux transportation by nucleotide efflux transporters (Boularan and Gales 2015). PDEs are encoded by twenty-one different genes and classified into eleven families. They are made up of two catalytic domains. Eight families of PDEs have been reported to be expressed in heart: PDE 1, 2, 3, 4, 5, 7, 8 and 9 (Omori and Kotera 2007, Boularan and Gales 2015). The important role of PDEs in forming the intracellular cAMP gradient in rat neonatal cardiomyocytes was first elucidated with the help of imaging approaches where it was also demonstrated that the gradient of cAMP might also activate PKA molecules (Zaccolo and Pozzan 2002). Certain PDE isoforms are responsible for selectively hydrolysing cAMP to 5-AMP, or in other words degrading it (Perino, Ghigo et al. 2012). Figure 1.8 shows the structure of PDEs.

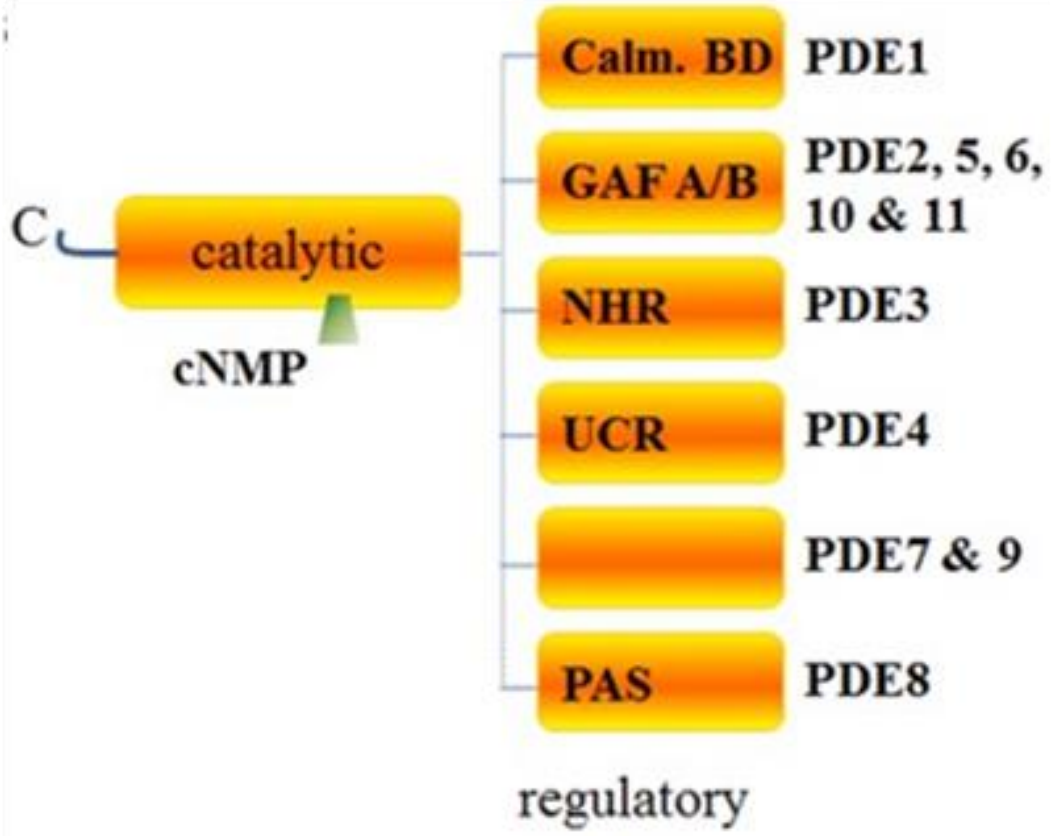


Figure 1.8: The structures of PDEs

The catalytic domain shown on the figure is conserved between all PDEs, whereas the regulatory domain varies between them. Calm BD: Binding site of calmodulin; GAF: cGMP-specific PDE, adenylate cyclase and FhIA; NHR: N-terminal; UVCR: Upstream conserved region; PAS: Per-ARNT-Sim (Boularan and Gales 2015)

One of the other ways by which the concentration of intracellular cAMP is controlled is its extrusion out of the cells via a transport system known as Multi Drug Resistant Protein (MRP). This transporter belongs to the super family of ATP-Binding Cassette (ABC) transporters (Cheepala, Hulot et al. 2013). MRP4 which is the most abundant isoform in the plasma membrane of cardiomyocytes (Sassi, Abi-Gerges et al. 2012) has been documented to regulate cAMP homeostasis in cardiac myocytes by efflux transportation. MRP4 deficient adult rat cardiomyocytes showed an increase in cAMP formation and were more prone to cardiac hypertrophy compared the sham group, an effect which was then compensated by an increase in PDEs expression (Sassi, Abi-Gerges et al. 2012).

cAMP signalling exerts its effects via different effectors. Its production activates two main downstream effectors: Protein Kinase A (PKA); and Exchange Protein Activated by cAMP (EPAC).

1.14.3 cAMP/PKA/EPAC signalling

Following β-adrenergic receptor stimulation (sympathetic) of heart cells, cAMP levels increase and activate cAMP dependent PKA/EPAC pathways to increase the force of contraction. This occurs as a result of PKA phosphorylating target proteins including Ca^{2+} channels and RyR to increase cytosolic Ca^{2+} which will activate greater interaction between actin and myosin because more Ca^{2+} would bind to troponin C during the systolic phase, promoting higher force of contraction (Cazorla, Lucas et al. 2009, Grimm and Brown 2010).

1.14.3.1 Protein kinase A

In an inactive state, PKA consists of a complex of two catalytic subunits and two regulatory subunits. The binding of two cAMP molecules to each regulatory subunit alters their conformation and liberates the catalytic subunits (which are now active) allowing them to phosphorylate their target proteins. Also, PKA phosphorylates other proteins that can accelerate relaxation which include phospholamban (PLN) (Figure 8).

On the other hand, phosphorylation of troponin I (which is the inhibitory unit of troponin complex) via PKA activation, enhances the relaxation during diastolic phase. It has been suggested that, phosphorylation at these sites could be the crucial mechanism responsible for cardiac relaxation and contraction (Okumura, Fujita et al. 2014)

In addition to altering excitation-contraction coupling, the activated PKA catalytic subunits enter the nucleus, where they phosphorylate a transcription factor known as cAMP Response Element Binding protein (CREB) at different phosphorylation sites, such as serine133 (Delghandi,

Johannessen et al. 2005, Sassone-Corsi 2012). Phosphorylated CREB then recruits a CREB binding protein forming a complex, which by binding to a specific regulatory region that is present in the promotor known as CREB Regulatory Element (CRE) regulates the transcription of the specific target genes (Figure 6), such as: cAMP Responsive Element Modulator (CREM), which is involved in insulin secretion and spermatogenesis; Hyaluronic Acid Synthase 1 (HAS1), which maintains tissue homeostasis and accelerates healing; and Nuclear Receptor subfamily 4 group A member 1 (NR4A), which is involved in vascular cell proliferation (Martinez-Gonzalez and Badimon 2005, Dwivedi and Pandey 2008, He, Song et al. 2014, Salvi and Abderrahmani 2014, Siiskonen, Oikari et al. 2015)

1.14.3.2 Exchange protein directly activated by cAMP

More recently described cAMP sensors are exchange proteins activated directly by cAMP (EPACs), which were discovered in 1998 (Kawasaki, Springett et al. 1998). Unlike PKA, EPAC is not a kinase but is a guanine exchange factor (GEF), exchanging GDP (inactive form) to GTP (active form) for the Ras family of G proteins, Rap1 and Rap2 (Borland, Smith et al. 2009). Figure 1.9 demonstrates the activation of Rap.

There are two isoforms of EPAC proteins, EPAC1 and EPAC2. Whereas, EPAC1 is abundantly distributed in heart, blood vessels and kidneys, the expression of EPAC2 is more restricted to pancreas, adrenal glands and central nervous system (Bos, de Rooij et al. 2001).

EPAC1 is encoded by *RAPGEF3* gene and EPAC2 is encoded by *RAPGEF4* gene (Lezoualc'h, Fazal et al. 2016). EPAC1 and EPAC2 share similar motifs in their structures. They both have an N-terminal regulatory a C-terminal region (de Rooij, Rehmann et al. 2000), which contains the following domains: a dishevelled, Egr-10, pleckstrin (DEP) domain; and cyclic nucleotide binding (CNB) domain. DEP domain is involved in EPAC membrane localisation, whereas the CNB domain binds to cAMP with high affinity (Lezoualc'h, Fazal et al. 2016, Ramos and Antonetti 2017). On the other hand, the C-terminal catalytic regions contain the following domains: the Ras exchange motif (REM) domain; Ras associated (RA) domain; and a cell division cycle 25, CDC, homology (CDC25H) domain. The REM domain is need for biological responses, the RA domain is required for perinuclear localisation of EPAC and the CDC25H domain is necessary for RAP activation and nuclear localisation of EPAC (Lezoualc'h, Fazal et al. 2016, Ramos and Antonetti 2017). Importantly, EPAC2 has an extra CNB domain. This additional CNB domain has a lower affinity for cAMP and it is not essential for the activation of EPAC2 (Lezoualc'h, Fazal et al. 2016). Figure 1.9 demonstrates the domain structures of EPAC isoforms.

Elevated cAMP interacts with the phosphate binding cassette of Cyclic Nucleotide-Binding Domain (CNBD-B) of EPAC. This interaction results in a conformational change of EPAC (Fujita, Umemura et al. 2017), liberates its catalytic domain and catalyses the exchange of Rap GDP (inactive form) for Rap GTP (active form). Figure 1.9 shows the activation of EPAC in response to elevated levels of cAMP.

The discovery of EPAC, as a cAMP sensor, raised questions regarding its mechanism of actions. PKA and EPAC are two distinctive cAMP signalling mediated pathways, the net effects of cAMP on cells could be mediated by PKA, EPAC or a cross talk between them (Cheng, Ji et al. 2008). Like PKA, EPAC signalling is controlled in a spatiotemporal manner by A kinase anchoring proteins (AKAPs), found in many different cell types (Poppinga, Munoz-Llancao et al. 2014). AKAP complexes highlight the importance of crosstalk existing between the effectors of cAMP. *In vitro* studies demonstrated opposing effects of PKA and EPAC activation on PKB regulation, where PKB is activated by EPAC stimulation, but PKA activation inhibits the activity of PKB (Mei, Qiao et al. 2002, Nijholt, Dolga et al. 2008).

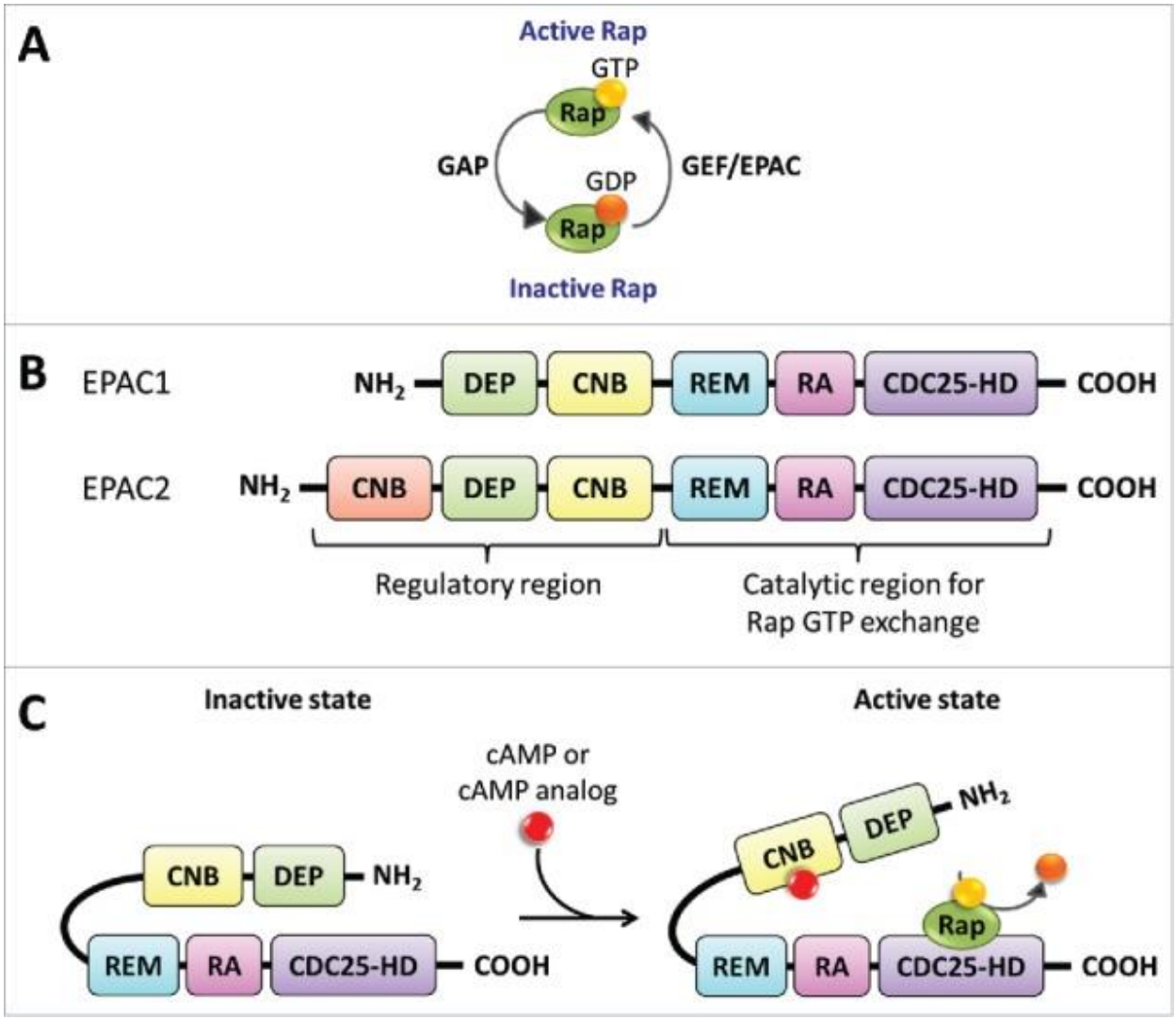


Figure 1.9: EPAC isoforms structures and their activations in response to elevated cAMP

A. Rap proteins which are GTPases, exchange GDP to GTP. GAPs catalyse the formation of GDP from GTP (via hydrolysis) to inactivate Rap. In contrast, GEF proteins, such as EPACs, increase the exchange rate of GDP for GTP, enhancing the activation of Rap. **B.** EPAC1 and EPAC2 are the two isoforms of EPAC. The regulatory region of EPACs contains CNB and DEP domains. The catalytic region contains REM, RA and CDC25H domains. **C.** EPAC is normally inactive, with its regulatory region folded over blocking the function of catalytic region. Once the intracellular levels of cAMP increase, cAMP binds to CNB domain, which induces a conformational change, whereupon the regulatory region is folded back, allowing access of catalytic region to Rap GTPases. Active EPAC binds Rap GTPases catalysing the exchange of Rap GDP to Rap GTP (Ramos and Antonetti 2017).

1.14.4 Mechanisms underlying the anti-fibrotic effects of cAMP and its effectors

The anti-cardio-fibrotic effects of cAMP are brought about in cardiac fibroblasts via: inhibition of action of growth factors that promote cardiac fibrosis; decrease in the proliferation rate; decrease in the migration rate; reduction in their differentiation to their active (myofibroblast) form; promotion of apoptosis; and minimising their ECM production (Heusinger-Ribeiro, Eberlein et al. 2001, Liu, Thangavel et al. 2008, Kohyama, Yamauchi et al. 2009, Sandbo, Kregel et al. 2009, Zhang, Yun et al. 2011, Insel, Murray et al. 2012).

Intracellular levels of cAMP can be elevated via different means, such as ligand stimulation of GPCRs (agonists or antagonists) coupled to activation of ACs, direct activation of ACs with forskolin, use of cAMP analogues (which diffuse into the cells) and inhibition of PDEs (Insel, Murray et al. 2012).

Studies carried out in rat cardiac fibroblasts suggested cAMP elevation as a potential way of inhibiting ECM production and collagen synthesis induced by TGF-β. The effect was accompanied by reduction in the protein levels of α-SMA, following the blockade of cardiac myofibroblast activation (Masur, Dewal et al. 1996, Swaney, Roth et al. 2005, Yokoyama, Patel et al. 2008). Further investigations implicated EPAC1 as a main reason for reduction in collagen synthesis (Yokoyama, Patel et al. 2008).

Further studies on human cardiac fibroblasts demonstrated that downregulation of cAMP (via inhibiting ACs) increased the levels of fibrotic proteins, such as collagen 1, PAI 1 and α-SMA. In contrast, elevation of the cAMP levels inhibited the scarring phenotypes of human cardiac fibroblasts (Clancy, Zheng et al. 2007).

The proliferation rate of cardiac fibroblasts is also regulated by cAMP signalling pathways. For example, cAMP production via the stimulation of A2BRs inhibits the proliferation of rat cardiac fibroblasts (Phosri, Arieyawong et al. 2017). Moreover, cAMP has been reported to have apoptotic effects on mice cardiac fibroblasts, an effect that was mediated by PKA activation (Zhang, Yun et al. 2011).

Signalling by cAMP has effects on fibroblast migration too. Investigations carried out on rat cardiac fibroblasts displayed complex effects of cAMP signalling on migration: agents that increase the levels of cAMP inside cardiac fibroblasts are pro-migratory at low concentrations, but have anti-migratory effects at higher concentrations (Yokoyama, Patel et al. 2008). PKA activation is documented to downregulate the migration rate of cardiac fibroblasts, whereas activation of EPACs promoted the migration of cardiac fibroblasts (Yokoyama, Patel et al. 2008).

1.15 Rationale for thesis and hypothesis

Cardiac fibrosis is a key aspect of heart failure, which is a prevalent, life-threatening condition for which current treatments are inadequate. Proliferation and migration of cardiac fibroblasts promote cardiac fibrosis after any type of damage, including post myocardial infarction and because of other cardiomyopathies that lead to heart failure. Whilst the current generation of anti-cardiac fibrosis drugs, such as ACE inhibitors (e.g. lisinopril), have beneficial effects, they only have modest effects on mortality but can also exert negative side effects (Packer, Poole-Wilson et al. 1999). Therefore, identifying signalling pathways that selectively inhibit cardiac fibroblasts proliferation and migration could lead to significantly improved treatments. This thesis focuses on the cAMP signalling pathway, which potently inhibits cardiac fibroblasts proliferation and migration *in vitro*. Crucially, cAMP also induces vascular protective effects in ECs, promoting barrier function and inhibiting inflammation and monocyte adhesion. Elevation of cAMP also inhibits VSMC proliferation by downregulating multiple key cell-cycle intermediates and may therefore retard atherosclerosis progression. However, cAMP elevation by PDE3 inhibitors has unwanted inotropic and chronotropic effects on the heart that have been associated with poorer survival in heart failure patients. Targeting downstream aspects of cAMP signalling that selectively reduce cardiac fibrosis would therefore be an advantage.

Recent data from our group link the anti-mitotic and anti-migratory activity of cAMP in SMCs to actin-cytoskeleton remodelling in response to inhibition of members of the Rho-GTPase family. We investigated whether similar mechanisms apply to cardiac fibroblasts and sought to elucidate the components of the regulatory pathways in sufficient detail to identify novel druggable targets.

In this thesis the following hypotheses were tested, cAMP elevating stimuli inhibit cardiac fibroblast proliferation and migration via remodelling of the actin-cytoskeleton and regulation of actin-sensitive pathways including MKL-SRF and YAP-TEAD.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Materials

2.1.1 General materials

All general reagents, except those stated otherwise, were purchased from Sigma-Aldrich Co. (Poole UK).

2.1.2 Cell Culture Reagents

Dulbecco's Modified Eagle Medium (DMEM), Advanced DMEM/F12 (1X), 1.0 g/L glucose, L-Glutamine (200 mM), foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), Dulbecco's Phosphate Buffered Saline (DPBS: Ca^{2+} and Mg^{2+} free) and 10x trypsin-ethylenediametetraacetic acid (EDTA) were acquired from Life Technologies.

2.1.3 Immunological reagents

Below are the details of primary and secondary antibodies used in our investigations (Tables 1 and 2).

Target protein	**Host species**	**Dilution**	**Supplier**	**Catalogue number**
EPAC1	Mouse	1:700	Cell signalling	4155
EPAC2	Mouse	1:700	Cell signalling	4156
MKL1	Mouse	1:1000	Cell signalling	14760
MKL2	Mouse	1:1000	Cell signalling	14613
GAPDH	Mouse	1:10000	Millipore	MAB374
Beta-actin (β-actin)	Mouse	1:1000	Sigma	A5316
BrdU	Goat	1:1000	Sigma	B2531
Phospho-MYPT (Thr853)	Rabbit	1:1000	Cell signalling	4563
phospho-MYPT (Thr696)	Rabbit	1:1000	Cell signalling	5163

Table 1. Primary antibodies with their details

Target protein	Host species	Dilution	Supplier	Catalogue number
Anti-mouse IgG/HRP	Rabbit	1:8000	Sigma	P0161
Anti-rabbit	Goat	1:8000	Sigma	P0217

Table 2. Secondary antibodies with their details

2.1.4 Chemicals

Working concentrations of drugs were determined by dose-response optimisation experiments. Stimulations were performed in serum free DMEM (serum starvation media) unless otherwise stated with appropriate solvent controls. The details of the drugs are shown in table 3.

Compound	Supplier	Working concentration	Catalogue number	Solvent
Forskolin	Sigma	25 µM	F6886	DMSO
Db-cAMP	Sigma	200 µM	F5978	Water
BAY60-6583	TOCRIS	5 µg/ml	4472	DMSO
Latrunculin B	TOCRIS	0.5 µg/ml	3974	DMSO
Cytochalasin D	TOCRIS	2 µM	1233/1	DMSO
ROCK inhibitor (Y27632)	Cell signalling	10 µM	S1049	DMSO
CCG203971	CAYMEN	20 µM	15075	DMSO
MG-132	Cell signalling	10 µM	2194S	DMSO
PKA inhibitor (H89)	Sigma	20 µM	B1427	DMSO
Lactacystin	TOCRIS	10 µM	2267	DMSO
Acetoxymethyl ester of N 6-benzoyl-cAMP (6-BNZ-cAMP-AM)	Biolog	20 µM	B079	DMSO
8-(4-Chlorophenylthio) adenosine- 3', 5'-cyclic monophosphate, acetoxymethyl ester (8-CPT-cAMP-AM)	Biolog	20 µM	C056	DMSO
Verteporfin	Sigma	10 µM	SML0534	DMSO
I-942	MOLPORT	20 µM		DMSO
Cycloheximide	Sigma	100 µg/ml	C7698	DMSO
Entinostat (MS-275)	Selleckchem	5 µM	S1053	DMSO

Table 3. Details of the chemicals used in the experiments

2.2 Methods

2.2.1 Isolation and culture of primary rat cardiac fibroblasts, rat H9C2 cells and human cardiac fibroblasts

Male Sprague Dawley rat pups were killed by cervical dislocation in accordance with the Directive 2010/63/EU of the European Parliament, under schedule 1 of the United Kingdom Home Office Animal Scientific Procedures Act 1986 and performed in accordance with guidelines, regulations and approval of the University of Bristol. Approval was granted by the University of Bristol ethical review board. Cultures of rat cardiac fibroblasts were prepared as previously described (Sala-Newby, Freeman et al. 2003). Briefly, hearts from 2- to 3-day-old rats were digested with four cycles of incubation in 0.1% (v/v) trypsin containing 0.02% (v/v) EDTA in PBS. Digestion was stopped by the addition of FCS to 20% (v/v). The dispersed cells were resuspended in DMEM supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin, and 100 U/ml penicillin and plated for 1 hour to allow fibroblasts to adhere. The unattached myocytes were removed with two washes in PBS. Cardiac fibroblasts and neonatal myocytes were cultured in Advanced DMEM/F12 supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin, 10% (v/v) FCS and 2 mM L-glutamine.

Human cardiac fibroblasts were purchased from Promocell (Heidelberg, Germany) and cultured in DMEM/F12 supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin, 10% (v/v) FCS and 2 mM L-glutamine.

H9C2 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin.

2.2.2 Cell passage

On reaching confluence, cells were washed twice in PBS and incubated with 1x trypsin EDTA solution at 37^{o}C until cells had detached from the tissue culture flask. Trypsinisation was terminated by addition of an equal volume of 10% (v/v) FCS/DMEM and the resulting cell suspension was centrifuged at 259 g for 5 minutes. The cell pellet was resuspended in 10% (v/v) FCS/DMEM at 37^{o}C with 5% (v/v) CO_2, with regular renewal of medium. Cells of passage 2-19 were used for experiments.

2.2.3 Cell counting, cryopreservation and resuscitation

Cells were counted using a Neubauer haemocytometer and the concentration of cells per ml was calculated using the formula: Average cell count per 1 mm^2 = number of cells × 10^4 per ml. Prior to freezing, cells were re-suspended in 1 ml 10% (v/v) dimethyl sulfoxide (DMSO) in 10% (v/v)

FCS culture media and placed at -80^{o}C in a freezing chamber and cooled at -1^{o}C /min, before transfer to liquid nitrogen the following day. To resuscitate cells, cryovials were thawed rapidly at 37^{o}C and diluted 1:10 with 10% (v/v) FCS culture media. Cells were centrifuged at 259 g for 5 minutes and resuspended in growth medium before transfer to a culture flask.

2.2.4 Induction of cell quiescence

All cell types were serum starved by incubation for 24 hours with serum free medium, unless otherwise stated (DMEM containing 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin).

2.2.5 Molecular biology

The bench, instruments and racks involved in RNA or DNA studies were cleaned by 70% (v/v) alcohol and then RNaseZap or DNAZap (Ambion Inc., Cambridgeshire, UK) to remove contaminating nucleases and DNA. All tubes, pipette tips and bottles used in the studies were purchased from suppliers with guarantee of being nuclease-free.

2.2.5.1 Total RNA extraction and quantification

Total RNA was extracted from all cell types using the PureLink RNA Mini Kit (Ambion) as per manufacturer's instructions. RNA was eluted from purification columns in 40 µl of nuclease-free water. RNA was then quantified using Nanodrop spectrophotometer technology (LabTech; model number: ND-1000). RNA concentration was obtained by measuring absorbance at 260 nm and 260/230 and 260/280 ratios determined the extent of ethanol and protein contamination, respectively. Only samples with sufficient purity were used for subsequent experiments.

2.2.5.2 Reverse transcription

First-strand cDNA was synthesised from 150 ng of total RNA by random hexamer priming using Quantinova reverse transcription kit (Qiagen) according to manufacturer's instructions except that a 10 µl reaction was performed. cDNA was then diluted to 5 ng/µl by the addition of 20 µl RNAase free water and stored at -20^{o}C.

2.2.5.3 Quantitative PCR

Quantitative qPCR was performed using Rotor-Gene Q (Qiagen) with Lightcycler 480 SYBR Green 1 Master PCR system (Roche Diagnostics, Ltd). Briefly, 1 µl template cDNA from the reverse transcription reaction was mixed with gene specific primers (0.5 µM) and enzyme mixture as stated in the manufacturer's protocol. Samples were then subjected to a qPCR reaction shown in the table 4.

STEP	SUB-STEP	TEMPERATURE (^{0}C)	TIME
PRE-INCUBATION	-	95	5 min
AMPLIFICATION (x45 CYCLES)	Denaturation Annealing Elongation	95 62 72	20 sec 20 sec 20 sec

Table 4. Summary of the reactions needed in qPCR.

Cycles of Threshold (Ct) values were determined for each reaction. This represents the cycle number where the fluorescent signal from amplified PCR product reaches the threshold of detection and is thus a relative measure of target concentration. Ct values were then used to determine the differential expression of mRNA between control and treated conditions.

Fold change = $2^{-\Delta Ct}$ where ΔCt = Ct (treated) - Ct (control).

Melt curve analysis was used as an indicator of single product amplification, with the number of peaks directly equated the number of distinct amplicons. Data was analysed from reaction were only a single melt-curve analysis peak was observed.

2.2.5.4 Primer design

Primers for qPCR analysis were designed using the NCBI Primer online application (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). This application includes a BLAST analysis to refine primer selection and avoid targeting of non-specific products. Filters included a PCR product size of 90-150 base pairs, optimal melting temperature (Tm) of 65ºC, optimal primer size of 22 base pairs and requisite for exon-exon or exon-intron junctions for spliced and pre-spliced mRNA respectively. Further specifications of primer size and GC content were also included to ensure optimal specificity. Sequences of quantitative PCR primes are available in the Appendix.

2.2.5.5 Agarose gel electrophoresis of DNA

PCR products were isolated from other DNA molecules (e.g. template DNA) by agarose gel electrophoresis and purification of the DNA product from excised gel slices. Negatively charged DNA molecules migrate towards the anode when a voltage is applied across the gel. Mobility of a DNA fragment is inversely related to the size of the fragment and the concentration of the agarose

gel, but proportional to the applied voltage. The gel was prepared by addition of an appropriate amount of agarose to 1x TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.2 μg/ml ethidium bromide. Agarose was dissolved in a microwave oven, allowed to cool and solidified into a clean gel tray and comb apparatus. DNA samples were mixed with 1/6 volume of 6x loading buffer (30% (v/v) glycerol, 0.25% (v/v) orange G, 0.25% (v/v) xylene cyanol FF) and then loaded into the wells of the gel. Electrophoresis was performed in 1x TAE buffer at 75-150 V depending on the size of gel until optimal separation of the DNA segment of interest was obtained. Gels were viewed under UV light illumination. Digital images were acquired and densities of the bands on gels quantified using a densitometric program (Alphalmager v5.5, Alpha Innotec Co. San Leandro, CA, USA). Required DNA bands were excised from the gel using a sterile scalpel.

2.2.5.6 DNA restriction, digestion and ligation

Purified DNA fragments were digested with specific restriction endonucleases (New England Biolabs) to generate 'sticky ends' that facilitate their ligation into plasmid vectors with compatible sticky ends. DNA was mixed with 1 x of the appropriate enzyme specific buffer and digested at 37^{o}C for 8 hours. Digested DNA was purified using DNA clean up columns (Qiagen) according to the manufacturer's instructions or by agarose gel purification, as described in section 2.2.5.5. Purified digested DNA was the ligated into the desired vector using a rapid ligation kit (Roche) according to the manufacturer's instructions.

2.2.5.7 Transformation of competent bacteria

Plasmids containing cloned cDNAs generated by ligation reactions were amplified by transformation into chemically competent DH5α bacteria (Invitrogen Ltd., Paisley, UK). Renewable stocks of plasmids were also prepared by transformation of DH5α bacteria. Competent bacteria were removed from -80 ºC and thawed on ice. 10 ng of stock plasmid solution or 2 μl of a ligation reaction described in 2.2.5.6 was added into 20 μl of competent cells with gentle mixing. Cells were incubated on ice for 20 minutes followed by a 45 second heat shock in a 42^{o}C water bath. Cells were then returned to ice for 5 minutes. Cells were incubated with 250 μl of SOC medium (Invitrogen Ltd., Paisley, UK) at 37^{o}C with shaking for an hour to allow cells to recover. 50 μl of cells was spread onto LB/Amp agar plates (15 g/l bacto-agar, 10 g/l bacto-tryptone, 5 g/l NaCl, 5 g/l bacto-yeast extract and 100 μg/ml ampicillin, pH 7.5). Plates were inverted and cultured at 37^{o}C for 16 hours to allow colonies to develop. Isolated colonies were picked using sterile pipette tips and the presence of the cloned gene was confirmed by PCR and DNA gel electrophoresis.

2.2.5.8 Preparation of plasmid DNA

Each confirmed colony or bacteria from a glycerol stock was added into 5 ml LB broth (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl, 100 μg/ml ampicillin, pH 7.5) and the mixture was incubated overnight at 37^{o}C in a rotatory incubator. The pellet from 4 ml of the mixture was obtained by micro-centrifugation at 10000 g for 3 minutes and the plasmid DNA was extracted from the cell pellet using QIAprep Spin Miniprep Kit (Qiagen Ltd., West Sussex, UK) according manufacturer's instruction. Briefly, 4 ml of bacterial overnight culture was pelleted by centrifugation at 10000 g for 3 minutes then resuspended in 250 μl of resuspension buffer containing RNase. Cells were lysed, neutralised and centrifuged for 10 minutes at 10000 g. The resulting supernatant was transferred to a separate spin column, centrifuged briefly, washed and finally the DNA was eluted in 50 μL elution buffer. Plasmid DNA was quantified using the Nanodrop spectrophotometer. Large scale preparations of endotoxin-free plasmid were made using GenElute HP endotoxin-free plasmid maxiprep kits (Sigma).

2.2.5.9 Plasmids adenoviral vectors and gene silencing

Plasmid pSRF-luciferase (#219079) containing five copies of the serum response element (SRE; 5'-AGG ATG TCC ATA TTA GGA CAT CT-3') upstream of the firefly luciferase gene was obtained from Stratagene. CREB-luciferase plasmid (α168) was gifted by Prof. G. S. McKnight (University of Washington) and has been described previously (Mellon, Clegg et al. 1989). TEAD-LUC luciferase reporter gene containing eight tandem copies of a TEAD binding element and a minimal TNT promoter was obtained from Addgene.

EPAC1 promoter fragments were generated by PCR using KOD polymerase from human genomic DNA. The location of the 800 bp promoter fragment is chr12:48152755-48153554 (Hg19). The proximal TEAD element (5'-GCATTCCTC-3') was mutated to (5'-GCgTgtCTC-3') by PCR with a reverse primer incorporating the mutated element. Promoter fragments were cloned into the Nhe1 sites of the secreted nano-luciferase reporter gene plasmid pNL3.3 [secNluc/minP] (Promega). The synthetic TEAD luciferase reporter plasmid containing eight copies of a TEAD binding element has been described previously (Dupont, Morsut et al. 2011) and was obtained from Addgene (plasmid #34615). Luciferase reporter plasmid containing the minimal TNT promoter was generated by Bgl II and Kpn1 digestion of the TEAD-LUC vector to remove the eight TEAD elements, leaving just the minimal TNT promoter. Plasmid expressing dominant-negative TEAD in which the YAP-binding domain of TEAD is replaced with the repressor domain of Engrailed (TEAD-ENRD) was a gift from Prof Domenico Flagiello (University of Paris Diderot) and has been described previously (Malt, Cagliero et al. 2012). Adenovirus vectors expressing constitutively active YAP

(Ad:YAP_{S127A}), constitutively active TAZ (Ad:TAZ_{5SA}) or control virus lacking a transgene (Ad:Control) have been described previous (Kimura, Duggirala et al. 2016). Cardiac fibroblasts were infected with $1x10^7$ pfu/ml of recombinant adenovirus for 18 hours.

For gene silencing, cells were transfected with 100 pmoles of Silencer Select siRNA (Life Technologies) targeting rat MKL1 (s163756), rat MKL2 (ABX00O2), YAP1 (ID:s170198 and ID:s170200), TAZ/WWTR1 (ID: s148961) using the standard Nucleofector program A-024. Cells were stimulated with the indicated agents 24 hours post transfection. The next day cell lysates were assayed for firefly luciferase activity and cell culture media assayed for secreted nano-luciferase. Firefly luciferase was quantified using the luciferase assay system (Promega) and secreted nanoluciferase activity assayed using the NanoGlo assay system (Promega) according to the manufactures instructions using Glomax Discover luminometer (Promega).

2.2.5.10 Reporter gene assays

EPAC1 promoter activity was determined by quantifying the secreted nano-luciferase reporter activity in cells transfected with EPAC1-NLUC. Plasmid transfection was performed by electroporation of $1x10^6$ cardiac fibroblasts with 5 µg of plasmid DNA using an Amaxa Nucleofector-1.5 (program A-024). Cultured media were conditioned for the indicated times. 10 µl of this lysate was mixed with 90 µl of RNase free water and assayed for EPAC-NLUC activity using a white 96-well plate micro-titre dish and Glomax Discover luminometer (Promega) according to the manufacturer instructions.

SRF and TEAD promoter activities were determined by quantifying the firefly luciferase reporter activities in cells transfected with SRF-LUC and TEAD-LUC. Plasmid transfection was performed by electroporation of $1x10^6$ cardiac fibroblasts with 5 µg of plasmid DNA using an Amaxa Nucleofector-1.5 (program A-024). Cells were lysed in 100 µl of ice cold 1x Promega reporter gene lysis buffer. 10 µl of this lysate was mixed with 90 µl of RNase free water and assayed for luciferase activity using a white 96-well plate micro-titre dish and Glomax Discover luminometer (Promega) according to the manufacturer instructions.

2.2.5.11 Chromatin Immunoprecipitation (CHIP) assay

Rat cardiac fibroblasts (approximately $8x10^6$ cells) were washed in PBS and fixed in 11% (v/v) formaldehyde in PBS for eight minutes at room temperature. Formaldehyde was quenched by addition of glycine to a final concentration of 250 mM. Chromatin was prepared using the iDeal ChIP-qPCRkit (Diagenode) according to the manufacturer's instructions. Chromatin was sheared using 2.5 cycles (10 rounds of 30 seconds on and 30 seconds off per cycles) of sonication in a

Bioruptor sonnicator (Diagenode). Sheared chromatin was immunoprecipitated overnight at 4ºC with either 1 µg of anti-H3K27 acetylation antibody (#C15410174; Diagenode) or an equal amount of rabbit non-immune IgG (#C15410206; Diagenode) according to the iDeal Chip-qPCR kit instructions. Following washing and elution, immunoprecipitated EPAC1 promoter DNA was quantified using qPCR with primers (Forward 5'- CCT CTG GAC TTG GAC TGG TCA TGC-3' and Reverse 5'- GCC GAA TTC CAG CCA GAA CTG AGA-3')

2.2.5.12 $RAP1_{GTP}$ activity assays

Levels of cellular $RAP1_{GTP}$ were quantified using the Cell Signalling Active Rap1 detection kit according to the manufacturer's instructions. Briefly, cells were seeded at $5x10^6$ cells/well in 6 well plates. Cells were pre-treated with 25 µM forskolin for 24 hours, as indicated before an acute stimulation the following day. $RAP1_{GTP}$ present in cell lysates was bound to 20 µg of GST-RAL-GDS protein and affinity isolated using glutathione resin. Following washing to remove non-specifically bound protein, specifically bound $RAP1_{GTP}$ as eluted in Laemmli sample buffer and analysed for RAP1 protein levels by Western blotting.

2.2.5.13 Electromobility shift assays

Electromobility shift assays (EMSA) were performed using the Lightshift EMSA kit (Thermo Fisher), according to the manufacturer's instructions. Nuclear extracts from $5x10^6$ control or pRK-myc-TEAD1 (Addgene #33109) transfected cells were prepared using the NE-PER nuclear extraction kit (Thermo Fisher). Double stranded 5' biotinylated probes corresponding to the wild-type or TEAD element mutant human EPAC1 promoter TEAD element (Wild-type sense 5'-Biotin-TTC CCC TAC GCA TTC CTC TAC CGT AA-3'; Wild-type antisense 5'-Biotin-TTA CGG TAG AGG AAT GCG TAG GGG AA-3'; TEAD-mutant sense 5'-Biotin- TTC CCC TAC GCg Tgt CTC TAC CGT AA-5' and TEAD mutant antisense 5'-Biotin-TTA CGG TAG Aga cAc GCG TAG GGG AA-3') were synthesised by Sigma Aldrich. 20 fmol of double stranded DNA oligo was used in 20 uL binding reactions according to the Lightshift assay kit protocol. DNA complexes were resolved on 5% (v/v) non-denaturing polyacrylamide/0.5xTBE gels at 100V. Oligonucleotides were transferred to Hybond-N nylon membrane (Amersham) in 0.5 x TBE at 400 mA for 1 hour at 4ºC. Oligonucleotides were detected using the Lightshift kit Extravidin-HRP detection system.

2.2.6 Visualisation of F-actin fibres

For detection of F-actin cells were cultured on sterile glass 13 mm diameter coverslips. F-actin stress fibres were detected by staining with Alexa-fluor-488 labelled phalloidin (Life Technologies).

Briefly, cells were fixed in 4% (v/v) paraformaldehyde for 10 minutes at room temperature before being permeabilised in 0.1% (v/v) Triton-X-100 in PBS for 5 minutes. Cells were then stained with phalloidin for 30 minutes in accordance with the manufacturer's instructions. Following staining, cells were washed in PBS and mounted onto glass microscope slides in Slow-Fade Gold Antifade mounting medium (Life Technologies). Images were acquired using an Olympus fluorescence microscope using an oil immersion x50 objective.

2.2.7 Visualisation of GFP-MKL in cardiac fibroblasts

Cardiac fibroblasts ($1x10^5$) were seeded in 24 well plate dishes. The next day, cells were infected with $1x10^7$ρfu/ml Ad:GFP-MKL1 in the presence of 10% (v/v) serum. The next day, cells were serum starved for 24 hours and then stimulated with specific cAMP elevating stimuli and/or mitogens for indicated times (as mentioned in each figure legend). Cellular localisation of GFP-MKL1 was quantified by capturing live cell fluorescence images using a EVOS live cell imaging system. Cells were counted as having nuclear MKL1 if the nuclear GFP signal was visibly more intense than the cytoplasmic signal. In contrast, cells were counted as having cytosolic MKL1 if the cytosolic GFP signal was visible more intense than the nuclear signal.

2.2.8 Quantification of F-actin:G-actin ratio

F-actin and G-actin were separated by Triton-X-100 solubility essentially as previously described (Parreno, Raju et al. 2014). (Parreno, Raju et al.) with slight modifications. Following treatment, G-actin was extracted by incubation with slight agitation at room temperature for 5 minutes in G-actin extraction buffer (PBS, 10% (v/v) glycerol, 0.1% (v/v) triton X-100, 1 mM ATP and complete protease inhibitor). Samples were centrifuged at 15,000 g at 4^{o}C for 5 min. The supernatant (soluble G-actin) was collected. Titon-X-100 insoluble material (F-actin) remaining in the wells and pelleted from the soluble fraction was lysed in reducing Laemmli SDS sample buffer. Samples were analysed by western blotting using a β-actin specific antibody (Sigma).

2.2.9 Western blotting

2.2.9.1 Preparation of cell lysates

Cells were seeded either at 5 x 10^5 cells in 6 well plates, or at 2.5 x 10^5 in 12 well plates and incubated overnight in growth medium at 37°C, 5% (v/v) CO_2. The following day cells were serum starved for 1 day. The next day, cells were stimulated with a specific agent for the indicated duration. Following stimulations, cells were washed in PBS and lysed in either 200 µL or 150 µL 1 x SDS lysis buffer respectively (2% (v/v) SDS, 50 mM Tris pH 6.8, 10% (v/v) glycerol) plus 25 µl/ml β-mercaptoethanol and 1 µL concentrated bromophenol blue. The lysis buffer was scraped around the

well to ensure complete lysis of all cells and then transferred to an eppendorf and repeatedly passed through a 200 µl pipette to shear genomic DNA and reduce viscosity. Following a brief vortex, lysates were placed on a 95^{o}C hot block for 5 minutes to reduce the samples.

2.2.9.2 Protein concentration quantification

The concentration of the protein lysate was determined using the micro BSA protein assay. A solution of 1 µg/µl BSA in from the 2 µl BSA standard provided with the BSA kit was made (60µl of the 2µl/BSA from the kit added to 60 µl of double distilled water in a 0.5 ml Eppendorf tube. The standards were made by mixing the reagents shown in the table 5. The standards were made by mixing the reagents shown in the table 5.

Standards	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1µg/µl BSA (aqueous)	0	0.5	1	2	3	4	6	8	10	15	25
Lysis buffer(µl)	5	5	5	5	5	5	5	5	5	5	5
Double distilled water (µl)	95	94.5	94	93	92	91	89	87	85	80	70

Table 5. Protein concentration quantification

Samples (lysates) were thawed from -20 °C storage. 5 µl of the lysates was added to 95 µl of water in a 0.5ml Eppendorf tube and mixed by repeated pipetting up and down. BCA assay reagent was prepared by mixing 500 parts of reagent A, 480 parts of reagent B and 20 parts of reagent C. An equal volume of reagent mixture was added to all the tubes (samples and standards), and samples mixed thoroughly by vertexing. Samples were incubated at 37°C for 20 minutes for a purple colour to be developed. 150µl of each sample was transferred to the wells of a 96 well plate the optical density read at 540 nm with a spectrophotometer (Bibby Scientific Limited). The protein concentration of each sample was determined by plotting the standard curve of BSA standard using Excel and linear interpolation. Equal concentrations of reduced and denatured protein samples were loaded into Mini-PROTEAN TGX 4-20% (v/v) gradient precast gels (BIO-RAD) and run in a Mini-PROTEAN Tetra Cell (BIO-RAD) filled with 1 x PAGE buffer [25 mM Tris-HCl (pH 8.3), 250 mM glycine, 0.1% (w/v) SDS] at 90 V until the bromophenol blue marker reached the bottom of the plates. A pre-stained molecular weight protein marker (Geneflow Limited) was also loaded to allow estimation of molecular weights.

2.2.9.3 Protein transfer and protein detection

Following electrophoresis, the gel plates were opened, the gel was trimmed and equilibrated in Tris-buffered saline-tween (TBST; 20 mM Tris pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20). The gel was then placed on a PVDF membrane and packed with filter paper and sponges to form a transfer sandwich structure. Figure 2.1 shows the arrangement of the transfer package. The gel membrane sandwich was placed vertically in a transfer tank, ensuring that the PVDF membrane was on the anode side of the gel. A magnetic stirrer and an ice block were placed inside the tank to maintain an even temperature and prevent overheating respectively. The electrodes were connected to the electric supply at a constant voltage of 100 V for 90 minutes at 4 °C. The membrane was then blocked with Tris-buffered saline-tween containing 5% (w/v) skimmed milk powder for at least 60 minutes at room temperature with rocking, to block non-specific binding. The blots were briefly washed in TBST and incubated with primary antibodies on a rocking table at 4^{o}C overnight. After removal of excess unbound primary antibody, blots were washed 4 times in TBST (each wash lasted for 5 minutes) and then incubated with 1:8000 HRP-conjugated secondary antibodies (Sigma) diluted in TBST containing 2.5% (w/v) skimmed milk powder for 60 minutes at room temperature, with rocking. This was followed by washing 4 times in TBST (each wash lasted for 5 minutes). To detect the bound antibodies, the blots were incubated with 1 ml Immobilon Western Chemiluminescent HRP substrate (Millipore) for 1 minute and luminescence was detected with high performance X-ray films (Amersham).

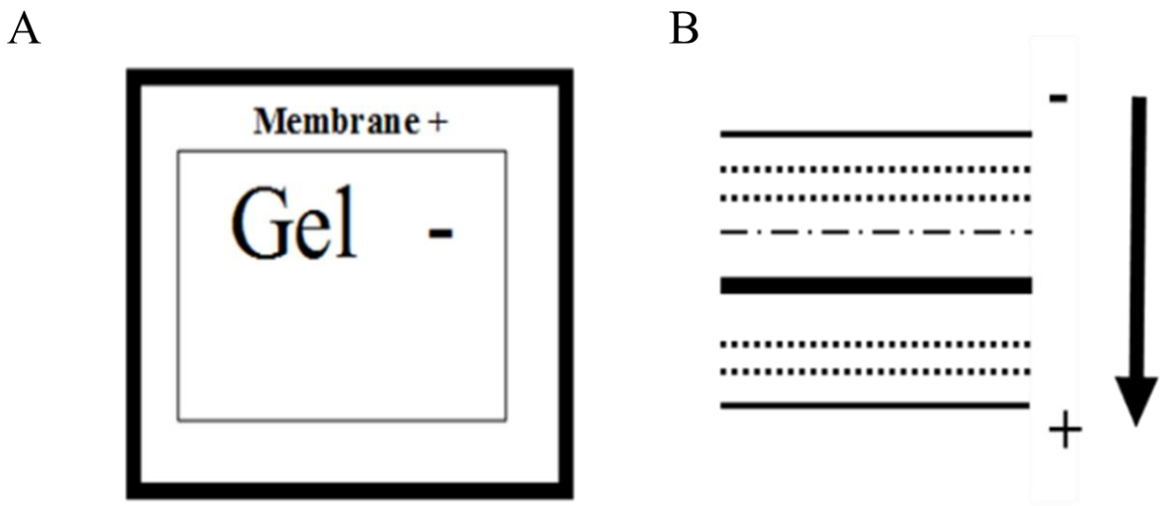


Figure 2.1: The arrangement of gel and membrane before going into the Bio-Rad semi-dry turbo blotter

The gel is placed on top pf the membrane because the proteins travelled from the Cathode to Anode (**A**). The assembly of the sandwich: from top to bottom: Cathode, 2 layers of filter paper, gel, PVDF membrane, 2 layers of filter paper and Anode (**B**)

2.2.10 BrdU proliferation assay

To quantify cell proliferation, a 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay (Sigma) was conducted. Rat cardiac fibroblasts were seeded on 13 mm gelatin coated glass coverslips (placed in the wells of a 24 well plate dish) were incubated overnight in 10% (v/v) FCS/DMEM. The following day cells were serum starved and stimulated as indicated. Rat cardiac fibroblasts were then treated with 10 μM BrdU for 6 hours and then washed in PBS and fixed in 70% (v/v) ethanol. After a brief wash in de-ionised water cell were incubated with pre-warmed 2.0 M HCl at 37^{o}C for 40 minutes. Following PBS washing, blocking buffer (5% (w/v) BSA/PBS) was added at room temperature for 30 minutes, washed again in PBS and incubated with anti-BrdU overnight at 4^{o}C. Washing in tap water caused the haematoxylin stained nuclei to stain blue. The following morning, wells were washed 5 times in PBS and treated with biotinylated goat-anti-mouse diluted 1:200 in 1% (w/v) BSA/PBS for 30 minutes at room temperature. After washing in PBS, extravidin-peroxidase (Sigma) diluted 1:200 in 1% (w/v) BSA/PBS was added for 30 minutes at room temperature. Wells were washed 4 times in PBS, rinsed in distilled H_20 and incubated with the HRP substrate SigmaFast 3,3'-diaminobenzidine (DAB, Sigma) until a brown precipitate was visible. Wells were then washed in distilled water and stained with the nuclear stain Mayer's Haemotoxylin for 30 seconds. Washing in tap water caused the haematoxylin stained nuclei to stain blue and then the coverslips were mounted onto glass slides coated with PVP mounting medium. Rat cardiac fibroblasts were imaged using an Olympus BX41 microscope and Q-capture pro 6.0 software. BrdU positive cells (brown) and negative cells (blue) were counted in 10 fields of view for each inhibitor concentration and the number of BrdU positive cells was expressed as a percentage of total cells counted.

2.2.11 Edu proliferation assay

To quantify cell proliferation, a 5-Ethynyl-2'-deoxyruridine (Edu) incorporation assay (Base Click) was conducted. Rat cardiac fibroblasts were seeded on 13 mm gelatin coated glass coverslips (placed in the wells of a 24 well plate dish) were incubated overnight in 10% (v/v) FCS/DMEM/F12. Cells were serum starved and stimulated as indicated. Rat cardiac fibroblasts were then treated with 10 μM Edu for 6 hours and then washed in PBS and fixed in 3.7% (v/v) formaldehyde1804 (made in PBS) for 15 minutes at room temperature. After a brief wash in 3% (w/v) BSA (made in PBS), rat cardiac fibroblasts were incubated with 0.5% (v/v) Triton X-100 (made in PBS) for 20 minutes at room temperature (to punch holes in the cell membrane and make the cells permeable). Following 3% (w/v) BSA in PBS wash, the reaction cocktail was made according to the manufacturer's instructions. The reaction cocktail was added to every well containing the cover slip and the plate was wrapped around an aluminium foil (to protect it from light and was placed on a rocker at room

temperature for 30 minutes. Following 3% (w/v) BSA in PBS wash, cells had their nuclei stained. Briefly, Hoechst 33342 was diluted in PBS (1:5000) and was added to every well containing the cover slip. Rat cardiac fibroblasts were then incubated at room temperature (wrapped around the aluminium foil) and rocked for 20 minutes. Following 3% (w/v) BSA in PBS wash, each well containing the cover slip was covered with 3% (w/v) BSA in PBS. Rat cardiac fibroblasts were imaged using an Olympus BX41 microscope and Q-capture pro 6.0 software. Edu positive cells (green) and all of the cells, including the proliferative and non-proliferative (blue), were counted in 5-6 fields of view for each condition and the number of Edu positive cells was expressed as a percentage of total cells counted.

2.2.12 Statistical analysis

Statistical analysis was performed using Graphpad Instat software. For experiments with only 2 conditions, data was quantified by a two-tailed paired t-test. For experiments comparing means of more than two groups, an ANOVA multiple comparisons test with Student Newman-Keuls post-test was used. In all statistical analysis an output of $p<0.05$ was accepted significantly different. Data is expressed as mean +/- standard error of the mean (SEM). N indicates the number of independent experimental repeats using distinct cell preparations.

CHAPTER 3:

EFFECTS OF cAMP STIMULATION ON CARDIAC FIBROBLAST PROLFIERATION, MIGRATION AND ACTIN CYTOSKLETON REMODELLING

3.1 INTRODUCTION

Myocardial injury or insult results in cardiomyocyte death, inflammation and pathological remodelling of the myocardium. This is often associated with excessive synthesis of extracellular matrix proteins by resident cardiac fibroblasts, resulting in myocardial fibrosis. This progressively reduces the compliance of the myocardium, which ultimately contributes towards the development of heart failure (Frangogiannis 2012). Heart failure results from various forms of cardiovascular pathologies, including pressure overload, aortic stenosis, hypertension, hypertrophic and post viral dilated cardiomyopathies a major cause of mortality worldwide, representing a major economic burden (Bharati and Lev 1995, Asbun and Villarreal 2006, Bernaba, Chan et al. 2010, Ashrafian, McKenna et al. 2011). Despite improvements in the therapeutic options for patients suffering from heart disease, it remains a major clinical problem and interventions designed to limit fibrosis are limited.

Increased proliferation and migration of cardiac fibroblasts contributes toward cardiac remodelling and repair following myocardial injury (Kong, Christia et al. 2014). However, excessive or prolonged proliferation of cardiac fibroblasts contributes towards the development of cardiac fibrosis and ultimately heart failure (Olson, Naugle et al. 2005, Rog-Zielinska, Norris et al. 2016). Proliferative expansion of the cardiac fibroblast population increases the number of ECM producing cells and enhances ECM deposition independently of increased ECM gene expression. The current generation of drugs used to treat patients suffering from cardiac fibrosis and heart failure, including beta blockers and PDE inhibitors, are designed to improve cardiac function. As such they do not treat the underlying cause of the pathology or limit fibrosis and can have serious side effects. For example, PDE inhibitors (milrinone) increased mortality in patients (Packer, Carver et al. 1991, Majure, Greco et al. 2013). Therefore, there is a clear clinical need to new to identify new drug targets for the treatment of myocardial fibrosis. To this end, it is essential that we fully characterise the signalling pathways that specifically regulate the proliferation and migration of cardiac fibroblasts (Fan and Guan 2016) to help develop novel therapies that may help limit the progression of cardiac fibrosis.

The cAMP signalling pathway has been proposed as a promising therapeutic target for the treatment of cardiac fibrosis, due to its well characterised anti-fibrotic effects in various tissues (Hamad, Zhu et al. 2012, Insel, Murray et al. 2012, Travers, Kamal et al. 2016, Vecchio, White et al. 2017). These anti-fibrotic effects may result, at least in part, from the ability of stimuli that elevate cAMP levels to reduce cell proliferation and migration in several different cell types. Recent studies have

suggested that stimuli that elevated levels of cAMP effectively reduce cardiac fibrosis, at least in part, by inhibiting the proliferation and migration rates of cardiac fibroblasts both *in vitro* and *in vivo* (Liu, Thangavel et al. 2008, Chan, Dusting et al. 2010, Miller, Cai et al. 2011, Insel, Murray et al. 2012). Moreover, activation of cAMP signalling pathways has been shown to have anti-fibrotic effects in several other tissues, such as liver (Windmeier and Gressner 1997), lung (Dunkern, Feurstein et al. 2007, Huang, Wettlaufer et al. 2008) and kidney (Kothapalli, Hayashi et al. 1998).

Translating cAMP elevating agents into effective therapies for cardiac fibrosis is challenging. Several lines of evidence demonstrate that, increased levels of cAMP, enhance cardiac contractility (ionotropy), rate (chronotropy) and the speed of conduction (Haikala, Kaheinen et al. 1997, Nakahara, Kawada et al. 1998, Sogabe, Sasaki et al. 2009, Zaccolo 2009), implying the cAMP elevating therapies would have undesirable off-target effects that affect myocyte function. Efficiently delivering cAMP analogues to cardiac cells is also challenging due to their poor membrane permeability (Fantidis 2010). Recently developed cAMP analogues modified with acetoxymethyl ester groups demonstrate improved membrane permeability. However, rapid esterase cleavage of this group *in vivo* limits their clinical usefulness (Saerens, Delvaux et al. 2010). The efficacy of cAMP elevation with the PDE inhibitor Milrinone in patients with severe chronic heart failure has been tested in clinical trials (Packer, Carver et al. 1991, Knight and Yan 2013, Leroy and Fischmeister 2018). However, despite its beneficial hemodynamic effects, long term therapy with milrinone has been shown to increase patient morbidity and mortality (Packer, Carver et al. 1991, Majure, Greco et al. 2013). Moreover, cAMP elevation induced by the GPCR agonist, adenosine, was documented to enhance the progression of liver, skin, lung and peritoneal fibrosis (Schneider, Lindsay et al. 2010, Cronstein 2011, Karmouty-Quintana, Zhong et al. 2012, Burnstock, Vaughn et al. 2014, Wirsdorfer, de Leve et al. 2016, Liu, Bing et al. 2019). Furthermore, adenosine administration demonstrated fatal consequences (Edlund, Straat et al. 1989, Straat, Henriksson et al. 1991, Mallet 2004, Rajkumar, Qureshi et al. 2017). As a result, there is a need to characterise specific downstream cAMP signalling pathways that could be targeted therapeutically to exploit the anti-fibrotic properties of cAMP while avoiding the undesirable effects of long term cAMP modulation on cardiac function. As mentioned previously, although stimuli that elevate levels of cAMP are believed to have the ability to reduce cardiac fibroblast proliferation (Dubey, Gillespie et al. 1997, Insel, Murray et al. 2012), the underlying signalling mechanisms are not yet fully understood. A better understanding of these mechanisms may help identify novel drug targets that allow us to harness he beneficial effects of cAMP for treatment of cardiac fibrosis and cardiac fibroblast proliferation, whilst avoiding the unwanted pleiotropic effects of cAMP on the heart.

Recent research in vascular smooth muscle cells has highlighted the important role of actin cytoskeleton remodelling in mediating the anti-proliferative and anti-migratory effects of cAMP. In these cells, cAMP-dependent inhibition of members of the Rho-GTPases has been implicated in mediating the anti-mitogenic and anti-migratory effects of cAMP in these cells (Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014, Smith, Hudson et al. 2017).

In this chapter, we test the hypothesis that cAMP-elevating stimuli inhibit cardiac fibroblast proliferation and migration and that this is associated with remodelling of the actin cytoskeleton

3.2 HYPOTHESIS

1. Elevation of cAMP inhibits proliferation and migration in cardiac fibroblasts
2. Elevation of cAMP inhibits actin polymerisation in cardiac fibroblasts

These hypotheses were tested via:

- BrdU and EdU to investigate the effects of different cAMP stimuli on the proliferation of cardiac fibroblasts
- Real-time scratch wound assay to determine the effects of cAMP stimuli on the migration of cardiac fibroblasts
- Quantification of F-actin and G-actin levels by fluorescent staining and selective Triton-X-100 solubilisation

3.3 RESULTS

3.3.1 Elevated cAMP inhibits serum stimulated proliferation in cardiac fibroblasts

We initially tested if elevated cAMP levels inhibited proliferation of rat cardiac fibroblasts *in vitro*. To elevate the intracellular levels of cAMP, cells were treated with 5 µg/ml of the A2BR synthetic agonist BAY60-6583, 200 µM of Dibutyryl-cAMP (cAMP analogue, Db-cAMP) or 25 µM of the adenylate cyclase activator, forskolin for 18 hours in the presence of serum mitogens. Proliferation was detected and quantified by incorporation of the thymidine analogue BrdU by pulse labelling the cells for the final 6 hours of the stimulation period. Stimulation of cardiac fibroblasts with BAY60-6583, Db-cAMP or forskolin significantly inhibited proliferation (to 6.55 ± 1.02%, $p<0.001$; 10.2 ± 0.82%, $p<0.001$; and to 2.16 ± 0.46%, $p<0.001$ respectively (Figure 3.1).

3.3.2 PKA and EPAC selective agonists synergistically inhibit serum-stimulated proliferation of cardiac fibroblasts

The cAMP sensors PKA and EPAC, have been implicated in mediating many of the biological effects of cAMP (Zaccolo 2009, Olmedo, Munoz et al. 2013, Fujita, Umemura et al. 2017, Laudette, Zuo et al. 2018, Zhang, Wang et al. 2019).

In order to investigate the relative contribution of PKA and EPAC signalling in the anti-mitogenic effects of cAMP in cardiac fibroblasts, cells were stimulated with the selective PKA agonist (6-BNZ-cAMP-AM) or the selective EPAC agonist (8-pCPT-2′-*O*-Me-cAMP-AM; abbreviated herein to 8-CPT-cAMP-AM), alone or in combination, for 18 hours, followed by staining of incorporated EdU. Due to serum esterase mediated cleavage of the acetoxymethyl ester group on these selective cAMP analogues, cell proliferation in this experiment was stimulated using 50 ng/ml of the physiological growth factor PDGF, instead of serum. PDGF-stimulated proliferation of cardiac fibroblasts was significantly inhibited by PKA activation (from 20.64±1.67% in controls to 14.0±2.19% EdU incorporation $p<0.01$) and by EPAC activation (to 14.8 ± 2.46% EdU incorporation $p<0.05$) alone. Furthermore, a combination of 6-BNZ-cAMP-AM plus 8-CPT-cAMP-AM acted additively to suppress PDGF-stimulated proliferation (to 9.77 ± 3.44% EdU incorporation $p<0.01$) of cardiac fibroblasts to levels that were significantly lower than in either 6-BNZ-cAMP-AM or 8-CPT-cAMP-AM stimulated cells (Figure 3.2).

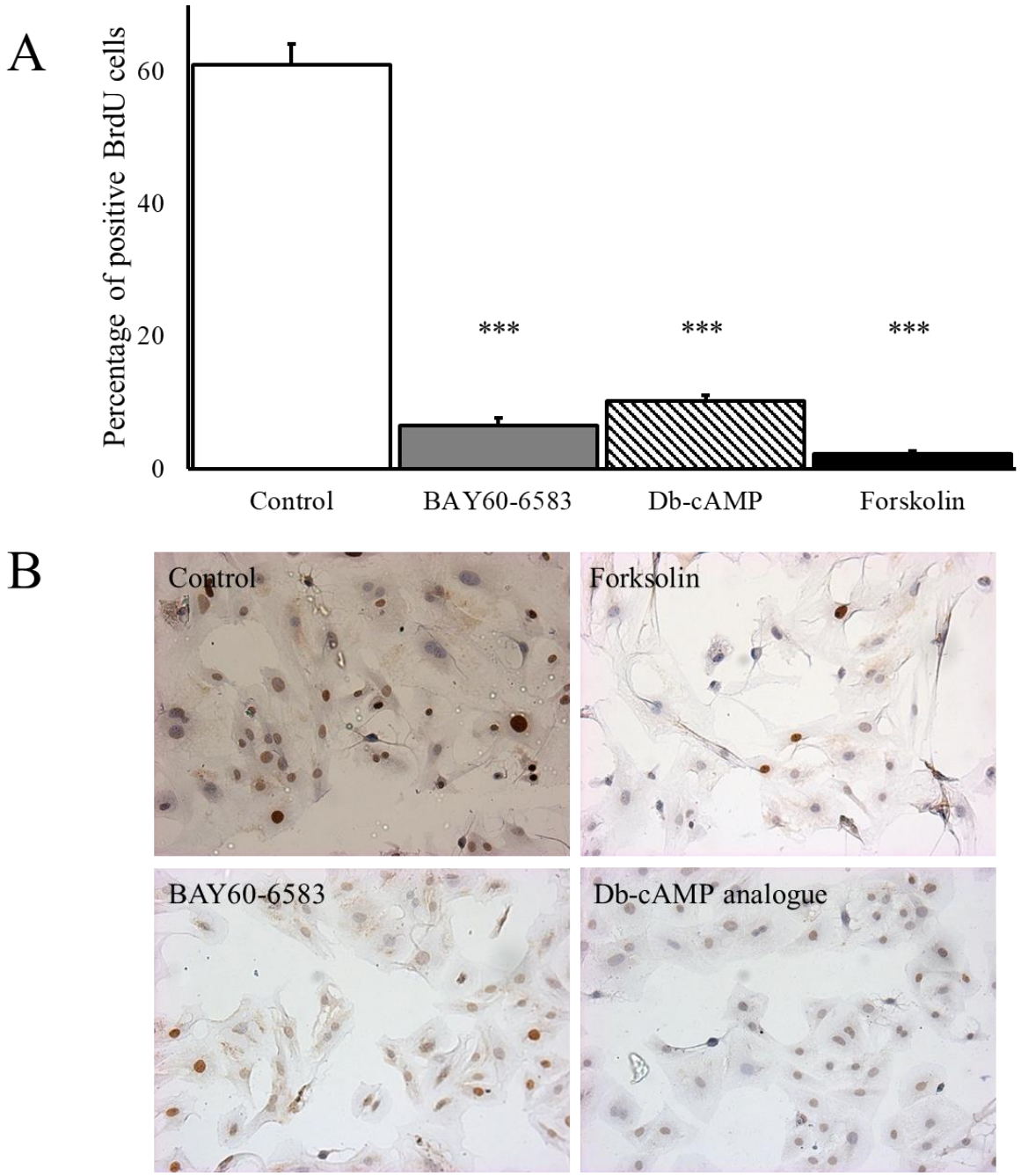


Figure 3.1: Elevated cAMP inhibits serum-stimulated proliferation of rat cardiac fibroblasts

Rat cardiac fibroblasts were stimulated for 18 hours with BAY60-6583 (BAY; 5 µg/ml), dibutyryl-cAMP (200 µM) or forskolin (25 µM) in 5% (v/v) serum, as indicated. Cells were labelled with 10 µM BrdU for further 6 hours and proliferation quantified by immuno-histochemical staining of incorporated BrdU. Representative images of each condition are shown below. ***: $p<0.001$ and Db-cAMP: dibutyryl-cAMP analogue (Db-cAMP analogue). Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

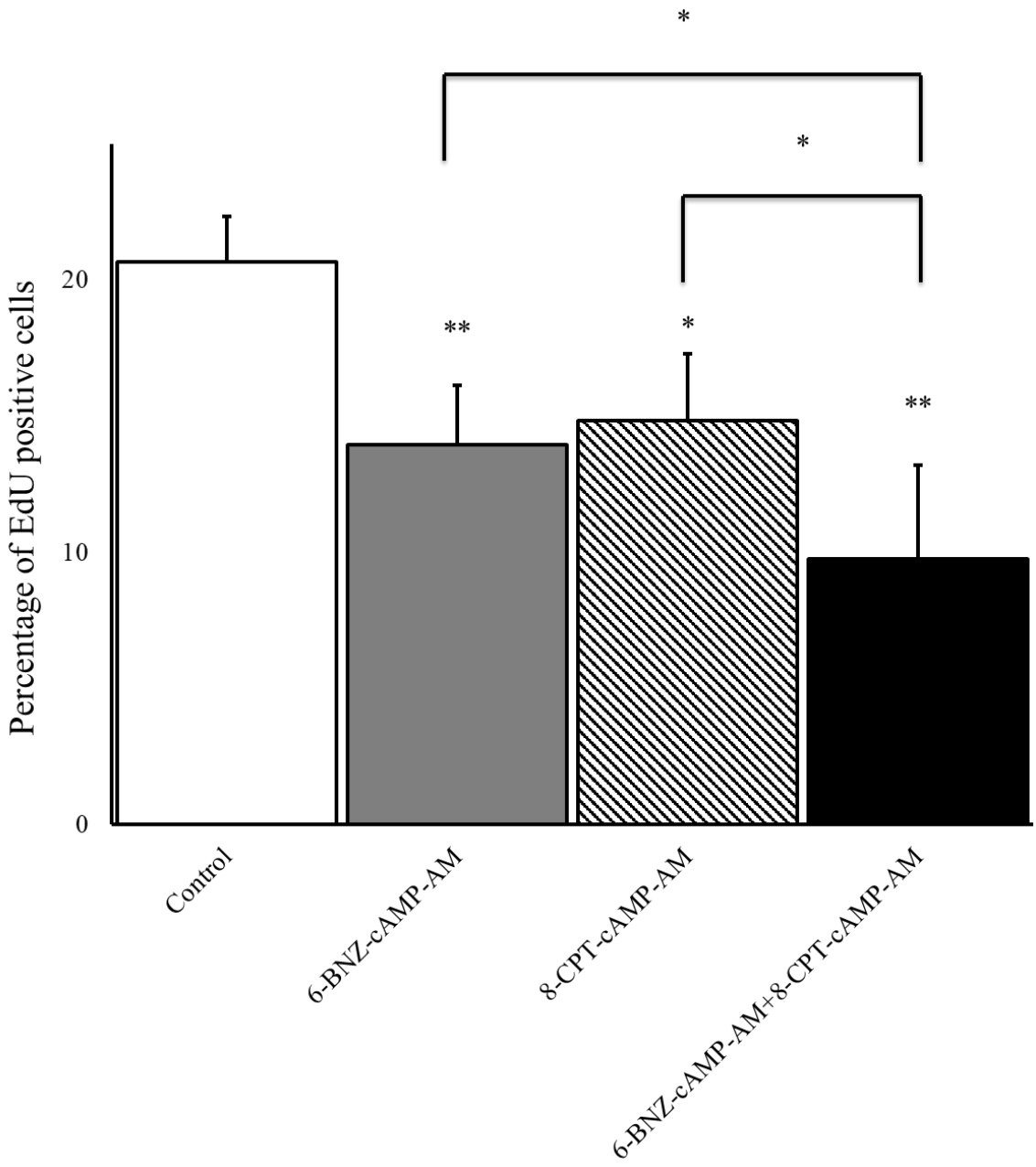


Figure 3.2: Elevated cAMP inhibits PDGF stimulated rat cardiac fibroblast proliferation

Rat cardiac fibroblasts were stimulated for 18 hours with 6-BNZ-cAMP-AM (20 µM) and 8-CPT-cAMP-AM (20 µM) in the presence of 50 ng/ml PDGF. Cells were labelled with 10 µM Edu for further 6 hours and proliferation was quantified as the percentage of by EdU-positive cells. PDGF: Platelet-derived growth factor; *: $p<0.05$ and **: $p<0.01$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

3.3.3 Elevated cAMP increases the migration of cardiac fibroblasts

Elevated cAMP signalling has been shown to inhibit cell migration in several cells types, including vascular smooth muscle cells (Hewer, Sala-Newby et al. 2011, Smith, Hudson et al. 2017). To investigate the effects of cAMP stimuli on migration of cardiac fibroblasts, cells were stimulated with 25 µM of forskolin or 200 µM of Db-cAMP analogue in the presence of 5% (v/v) serum and real-time migration rates were quantified over 24 hours using InnuCyte ZOOM scratch wound migration assays. Interestingly, elevation of cAMP with either forskolin or Db-cAMP analogue, resulted in a small increase of cardiac fibroblasts migration (Figure 3.3).

3.3.4 Effects of elevated cAMP levels on the morphology and actin cytoskeleton remodelling of cardiac fibroblasts

Whilst analysing the effects of cAMP on rat cardiac fibroblast proliferation and migration, it was noted that cAMP elevating stimuli induced a dramatic change in cell morphology similar to the stellate morphology previously reported in vascular smooth muscle cell (Pelletier, Julien et al. 2005, Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011, Smith, Hudson et al. 2017). To gain an insight into the possible functional significance of cAMP-induced morphological changes on proliferation and migration of cardiac fibroblasts, detailed time-course experiments were performed. Stimulation of serum starved cardiac fibroblasts with 25 µM forskolin or 5 µg/ml BAY60-6583 resulted in a rapid induction of a rounded, condensed stellate (star-like) cell morphology (Figure 3.4 and 3.6), characterised by reduced cell spreading (Figure 3.5 and 3.7). Phase contrast images were taken at regular time intervals and average cell area was determined using ImageJ software, from 10-16 fields of view per condition. Acquisition of stellate morphology, characterised by reduced cell spreading, was evident only after 20 minutes (from 0.040 ± 0.006 mm^2 in control to 0.014 ± 0.001 mm^2, $p<0.001$) for forskolin (Figure3.4 and 3.5) and after 40 minutes (from 0.039 ± 0.008 mm^2 in control to 0.0197 ± 0.002 mm^2, $p<0.05$) for BAY60-6583 (Figure 3.6 and 3.7).

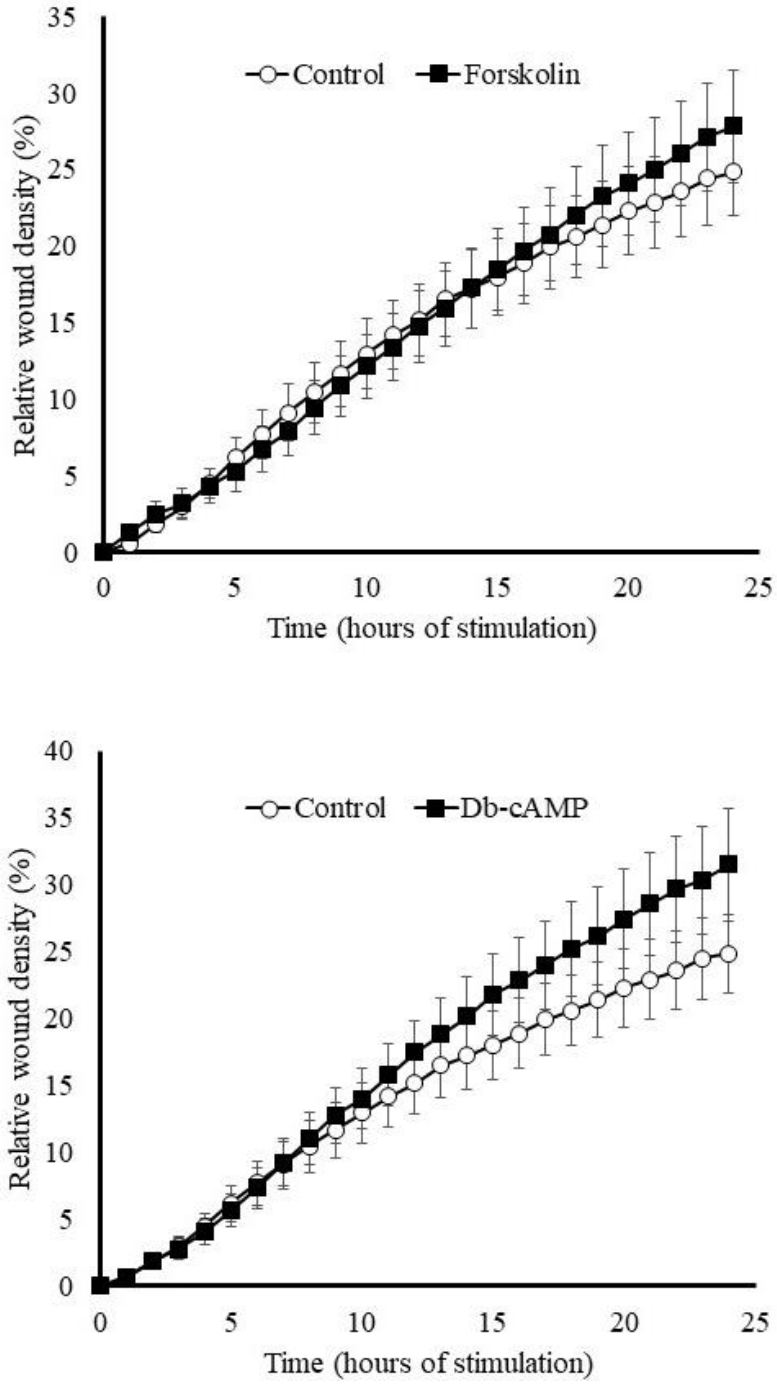


Figure 3.3: cAMP elevation does not have any effect on the migration of cardiac fibroblasts

Real-time scratch-wound migration analysis of rat cardiac fibroblasts stimulated with 25 µM forskolin (top panel) or 200 µM db-cAMP analogue (bottom panel) in 5% (v/v) FCS was performed using an IncuCyte ZOOM live-cell analysis system. FSK: forskolin; Db-cAMP analogue: dibutyryl-cAMP analogue. Data are expressed as mean ±SEM, n=6.

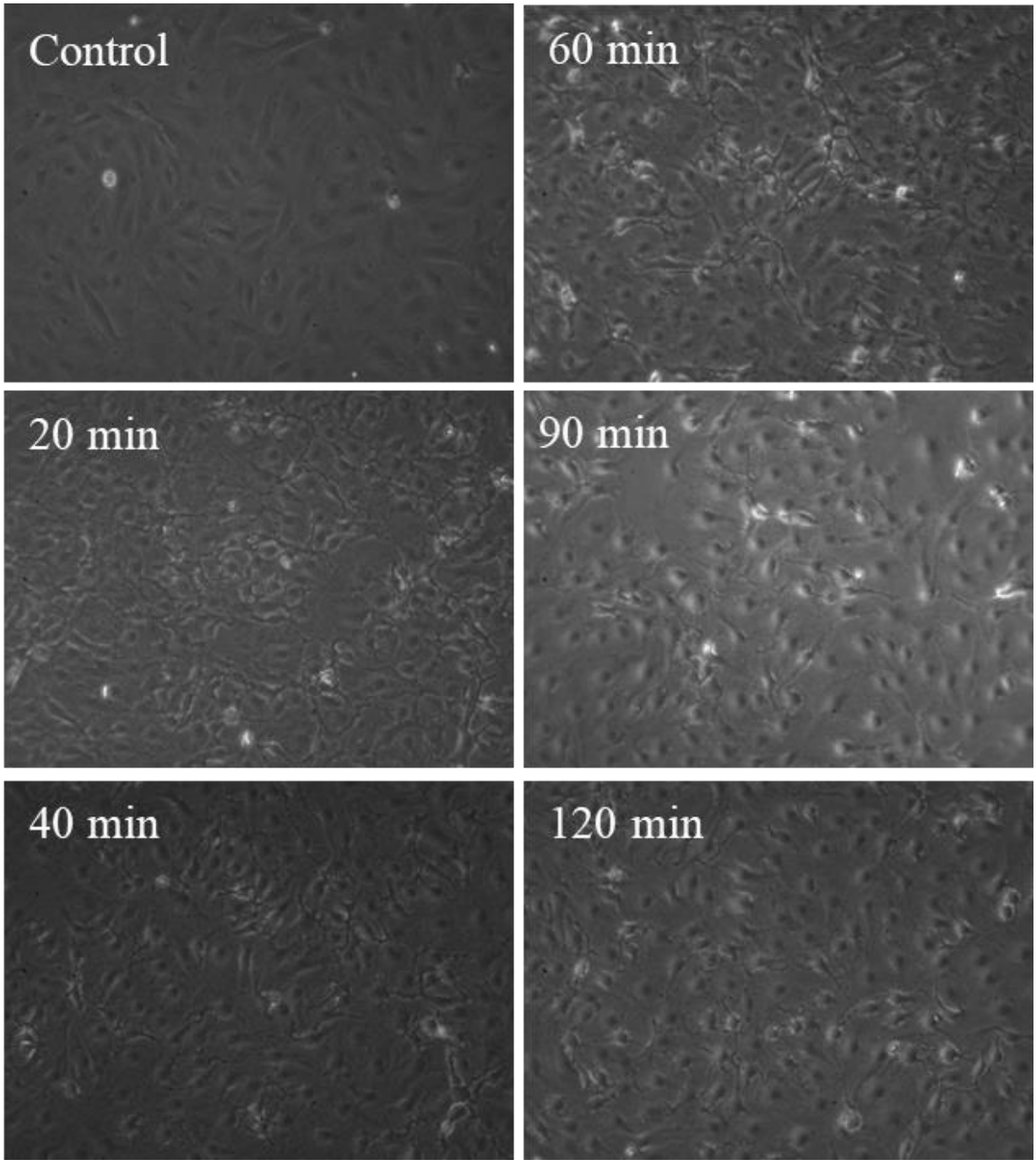


Figure 3.4: Forskolin induces morphological changes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated in serum-free conditions with 25 µM forskolin and phase contrast microscopy taken at the indicated times. Min: minutes.

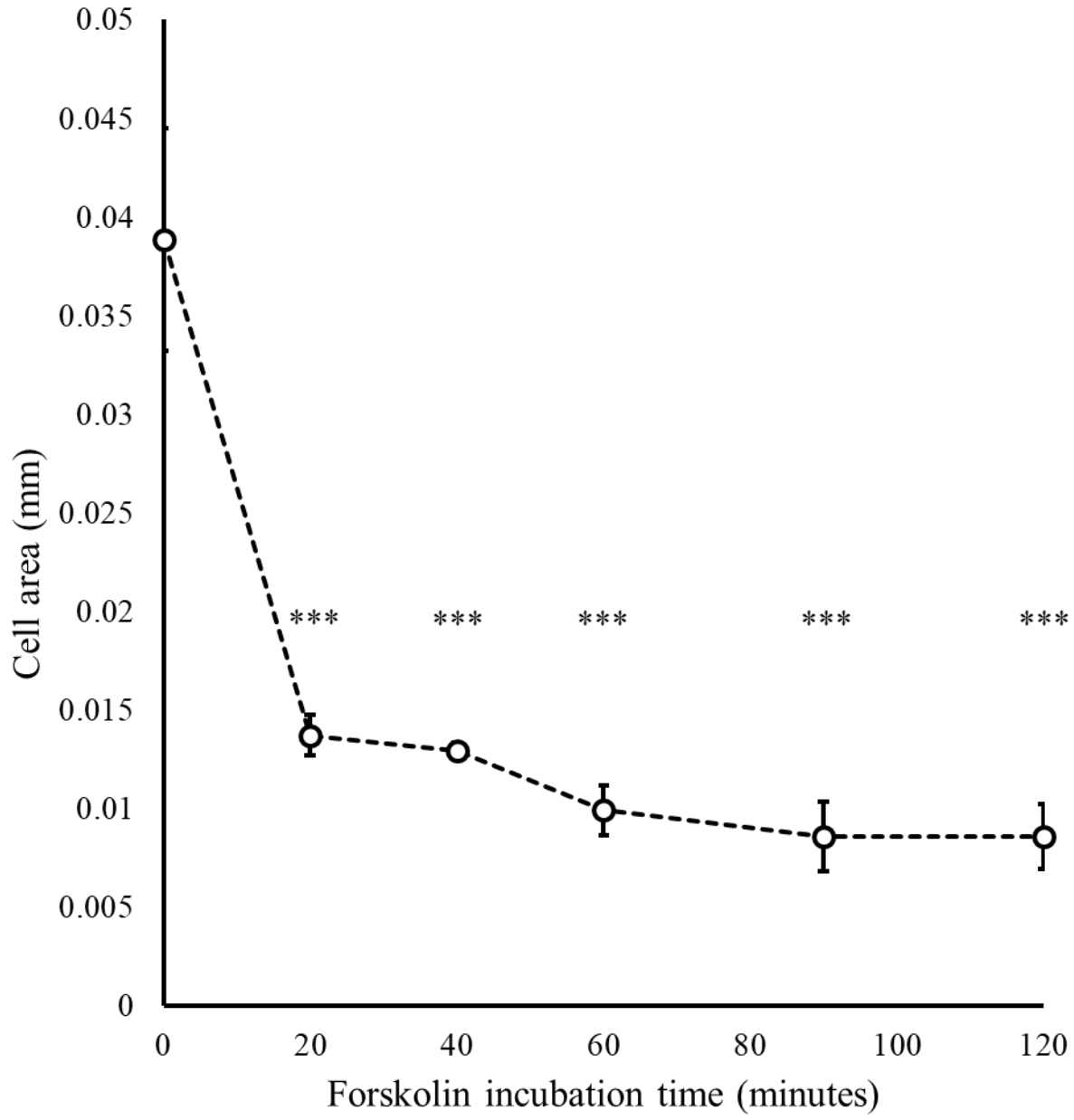


Figure 3.5: Forskolin inhibits cell spreading in cardiac fibroblasts

Rat cardiac fibroblasts were serum-starved overnight before being stimulated with 10% (v/v) FCS in the presence of 25 µM forskolin for the indicated times and total cell area assessed by image analysis of phase contrast images using ImageJ software. ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

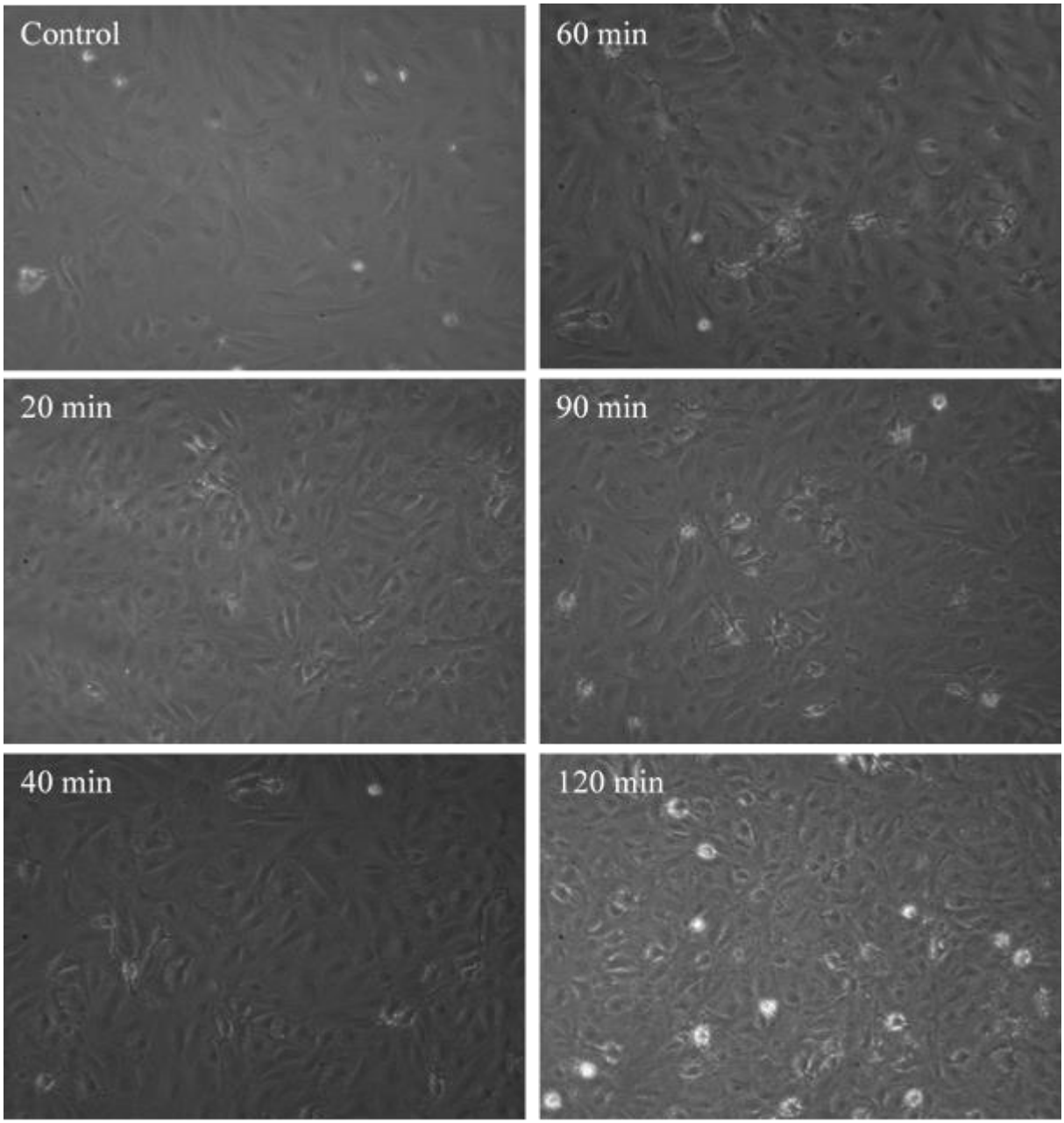


Figure 3.6: BAY60-6583 induces morphological changes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated in serum-free conditions with 5 µg/ml BAY60-6583 and phase contrast microscopy taken at the indicated times. Min: minutes.

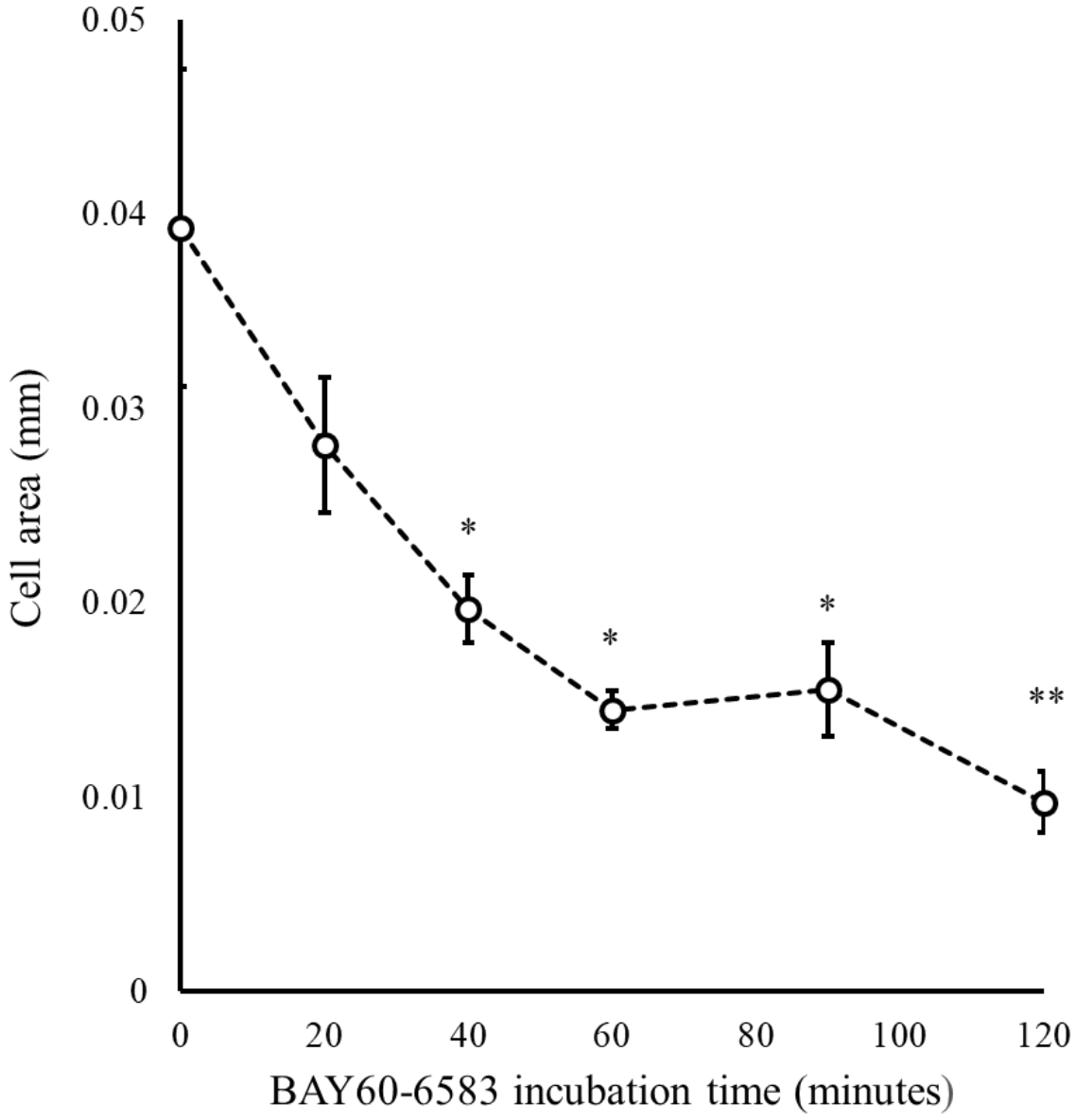


Figure 3.7: BAY60-6583 inhibits cell spreading in cardiac fibroblasts

Rat cardiac fibroblasts were serum starved overnight before being stimulated with 10% (v/v) FCS in the presence of 5 µg/ml BAY60-6583 for the indicated times and total cell area assessed by image analysis of phase contrast images using ImageJ software. *: $p<0.05$ and **: $p<0.01$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

3.3.5 PKA and EPAC specific activation synergistically inhibit cell spreading in cardiac fibroblasts

cAMP elevation activates both PKA and EPAC signalling. In order to determine which of these cAMP effectors is responsible for the cAMP-induced morphological changes, cardiac fibroblasts were stimulated with the cell permeable EPAC selective cAMP analogues, 8-CPT-cAMP-AM and I-942 alone, or in combination with the PKA selective agonist 6-BNZ-cAMP-AM. In cardiac fibroblasts, stimulation with the PKA selective agonist 6-BNZ-cAMP-AM alone induced a detectable change in morphology that was further enhanced by co-stimulation with 8-CPT-cAMP-AM or I-942 (Figures 3.8 and 3.9).

3.3.6 Elevated cAMP decreases F-actin and increases G-actin levels in cardiac fibroblasts

To determine the effects of cAMP elevation on actin cytoskeleton remodelling, cardiac fibroblasts were treated with either 25 µM forskolin or 5 µg/ml BAY60-6583 and stained with Alexa-fluor-488-conjugated phalloidin which detects F-actin fibres. Analysis of F-actin fibres in cardiac fibroblasts using Alexa-fluor-488-conjugated phalloidin staining demonstrated a rapid loss of F-actin stress fibres within 20 minutes of 25 µM forskolin stimulation. This loss of F-actin stress fibres persisted for at least 60 minutes (Figures 3.10 and 3.11). Loss of phalloidin stained F-actin fibres implies impaired actin polymerisation and increased levels of G-actin monomer. To further confirm these changes in the actin polymerisation in cardiac fibroblasts, levels of F-actin and G-actin were quantified using selective solubilisation of G-actin in Triton-X-100, as explained in the methods chapter. Consistent with phalloidin data indicating a reduction in actin polymerisation in response to elevated cAMP, stimulation of cardiac fibroblasts with forskolin or BAY60-6583 resulted in a significant reduction in the levels of F-actin and an increase in the levels of G-actin (Figure 3.12).

3.3. Y27632 induces morphological changes in cardiac fibroblasts

We have demonstrated that elevation of cAMP levels causes significant morphological changes, depolymerises of the actin cytoskeleton and inhibition of cardiac fibroblast proliferation, without affecting cell migration. Studies in other mesenchymal cell types have linked cAMP signalling with inhibition of the RhoA-ROCK pathway (Laudanna, Campbell et al. 1997, Tamma, Klussmann et al. 2003, Oishi, Makita et al. 2012, Akakpo, Musicki et al. 2017, Yu, Zhang et al. 2017). Previously, our group suggested that the effects of cAMP on proliferation and migration of vascular smooth muscle cells is due, at least in part, to its inhibitory effects on Rho GTPase signalling (Bond, Wu et al. 2008, Duggirala, Kimura et al. 2015, Kimura, Duggirala et al. 2016). In order to test this hypothesis, cardiac fibroblasts were treated with a pharmacological ROCK inhibitor (Y27632). Stimulation of cardiac fibroblasts with 10 µM Y27632 significantly downregulated the levels of

MYPT phosphorylated at Thr853, a well characterised ROCK substrate (Rana and Worthylake 2012) after 20 (to 0.033±0.01%, $p<0.001$), 40 (to 0.024±0.009%, $p<0.001$) and 60 (to 0.01±0.002%, $p<0.001$) minutes of incubation (Figure 3.13), confirming that Y27632 at this dose (10 uM) effectively inhibits ROCK signalling.

Given that RhoA-ROCK signalling has been implicated in regulation of the actin cytoskeleton (Narumiya, Ishizaki et al. 1997, Zeidan, Javadov et al. 2006, Amano, Nakayama et al. 2010, Gallo, Khan et al. 2012), we asked if inhibition of ROCK signalling induces a similar morphological change and actin cytoskeleton remodelling to that induced by cAMP elevation. Stimulation of serum starved cardiac fibroblasts with 10 µM Y27632 resulted in the rapid induction of rounded stellate morphology that was similar to that induced by cAMP elevating stimuli (Figure 3.14).

3.3.9 Y27632 inhibits actin stress fibre formation in cardiac fibroblasts

To determine the effects of ROCK inhibition on actin cytoskeleton remodelling, cardiac fibroblasts were treated with 10 µM Y27632 and stained with phalloidin, which binds specifically to F-actin stress fibres (Cooper 1987). Analysis of F-actin stress fibres in cardiac fibroblasts using Alexa-fluor-488-conjugated phalloidin staining demonstrated a dramatic and rapid loss of phalloidin stained F-actin fibres after stimulation with 10 µM Y27632, implying impaired actin polymerisation and increased actin depolymerisation. This is consistent with previous studies in vascular smooth muscle cells demonstrated by our group (Duggirala, Kimura et al. 2015, Smith, Hudson et al. 2017) (Figure 3.15).

3.3.10 Y27632 down regulates serum stimulated proliferation of cardiac fibroblasts

Signalling through the RhoA-ROCK pathway is known to regulate actin polymerisation (Zeidan, Javadov et al. 2006, Amano, Nakayama et al. 2010, Sit and Manser 2011).Therefore, we tested if cAMP-mediated inhibition of cardiac fibroblast proliferation is associated with the inhibition of RhoA-ROCK signalling. In order to test this hypothesis, cardiac fibroblasts were treated with 10 µM Y27632 in the presence of serum mitogens for 18 hours. Proliferation was detected and quantified by incorporation of the thymidine analogue BrdU by pulse labelling the cells for the final 6 hours of the stimulation period. Stimulation of cardiac fibroblasts with Y27632 significantly inhibited their proliferation (to 8.76±4.54%, $p<0.01$) (Figure 3.16).

3.3.11 Y27632 up regulates serum stimulated migration of cardiac fibroblasts

While several lines of evidence demonstrate an inhibitory role of ROCK inhibitors on cellular migration (Sadok, McCarthy et al. 2015, Wang, Yang et al. 2016, Guerra, Oliveira et al. 2017, Smith, Hudson et al. 2017), other studies imply that ROCK signalling is inhibitory to cells

migration, with ROCK inhibition enhancing the ability of cells to migrate (Yang and Kim 2014, Piltti, Varjosalo et al. 2015, Chang, Zhang et al. 2018). To investigate the effects of ROCK signalling on the migration of cardiac fibroblasts, cells were stimulated with 10 µM Y27632 in the presence of 5% (v/v) serum and real-time migration rates were quantified over 24 hours using IncuCyte ZOOM scratch wound migration assay. Stimulation of cardiac fibroblasts with Y27632 increased the migration of cardiac fibroblasts in real-time scratch wound assays (Figure 3.17).

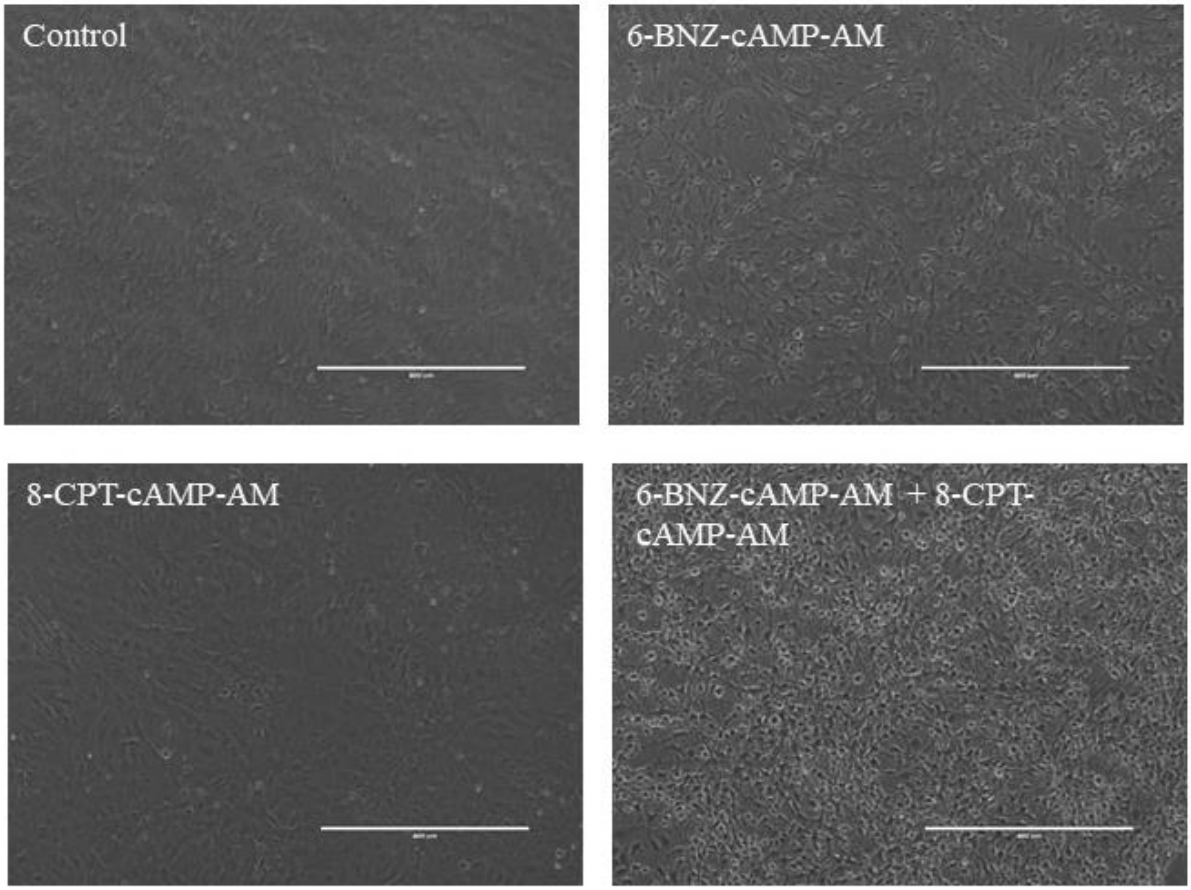


Figure 3.8: PKA and EPAC specific activation synergistically induce morphological changes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated in serum-free conditions with 20 µM 6-BNZ-cAMP-AM (PKA specific agonist) and 20 µM 8-CPT-cAMP-AM (EPAC specific agonist). Phase contrast microscopy was taken 2 hours after stimulation.

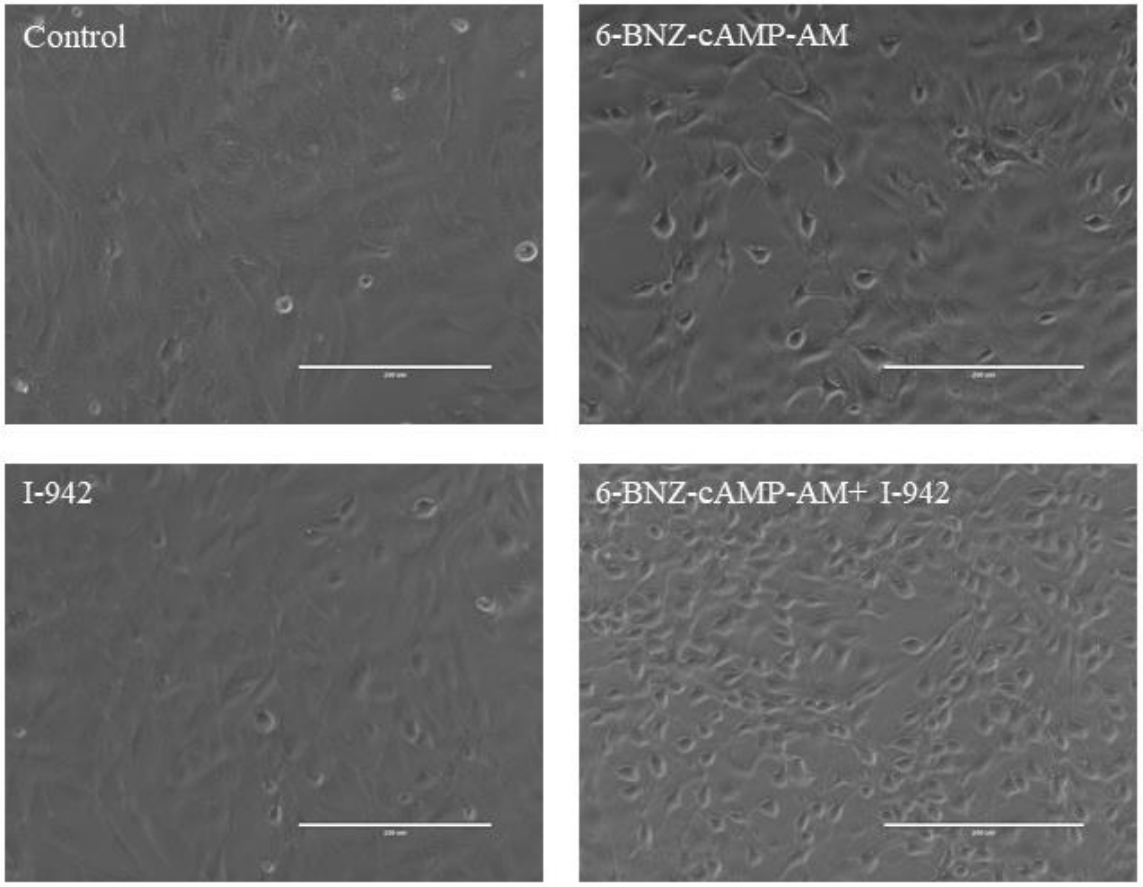


Figure 3.9: PKA and EPAC specific activations synergistically induce morphological changes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated in serum-free conditions with 20 µM 6-BNZ-cAMP-AM and 20 µM I-942. Phase contrast microscopy was taken 2 hours after stimulation.

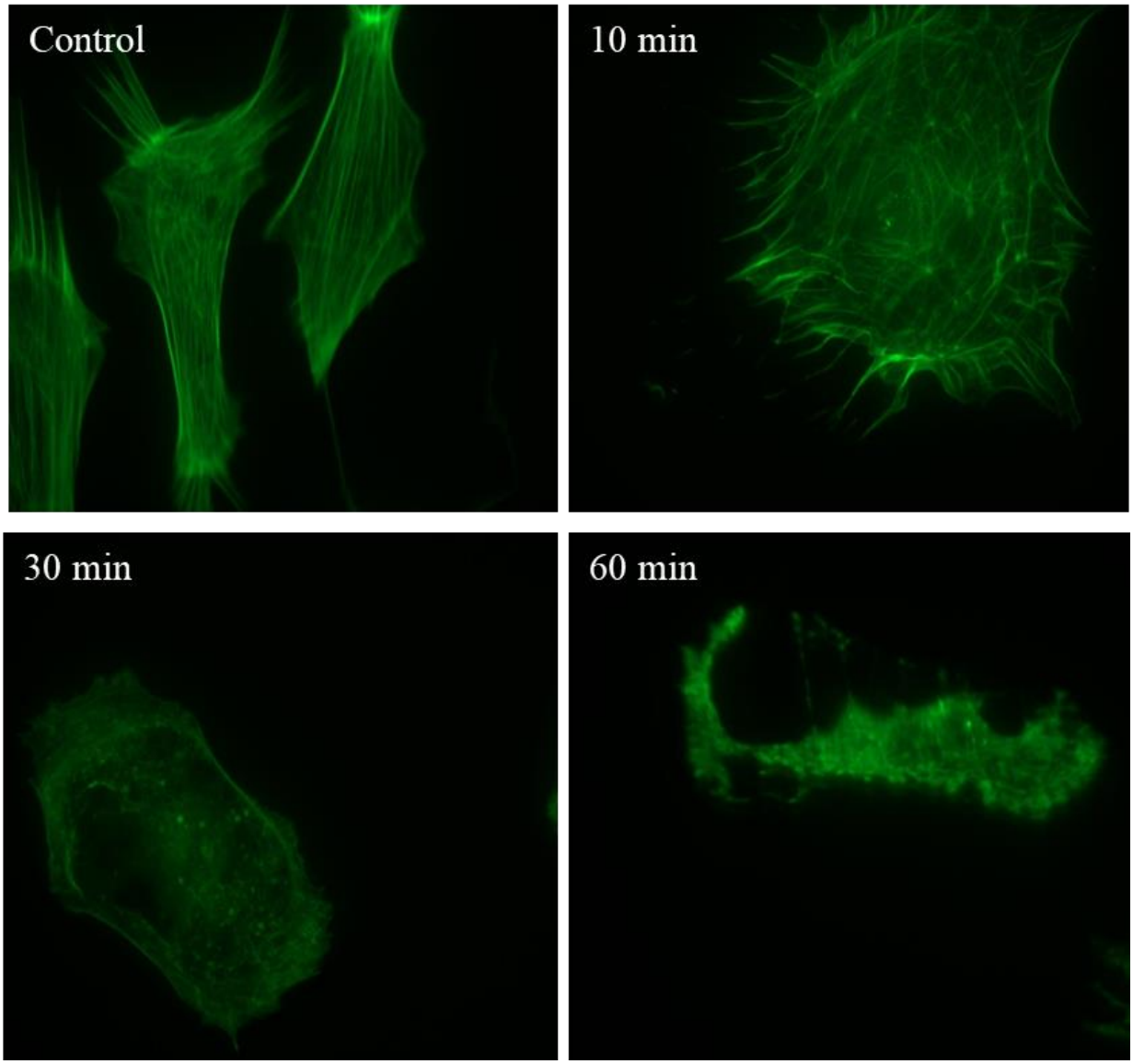


Figure 3.10: Forskolin stimulation disrupts the F-actin fibres in cardiac fibroblasts.

Rat cardiac fibroblasts were serum-starved for 4 hours before stimulation with 25 µM forskolin in serum free conditions for indicated times. Polymerised F-actin filaments were detected using Phalloidin staining of fixed cells. Representative images are shown.

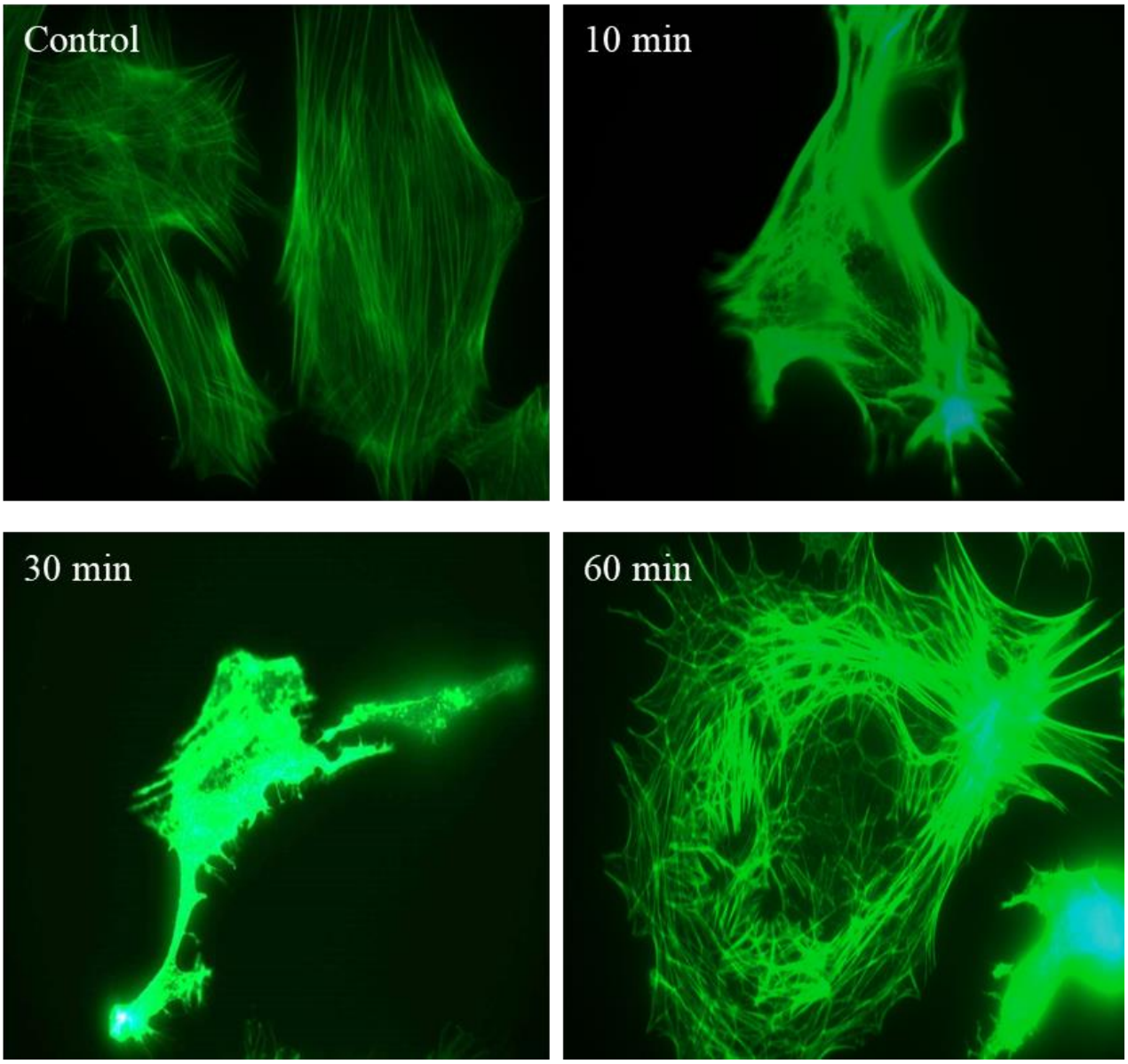


Figure 3.11: BAY60-6583 stimulation disrupts the F-actin fibres in cardiac fibroblasts.

Rat cardiac fibroblasts were serum-starved for 4 hours before stimulation with 5 µg/ml BAY60-6583 in serum free conditions for indicated times. Polymerised F-actin filaments were detected using Alexa-fluor-488 phalloidin staining of fixed cells. Representative images are shown.

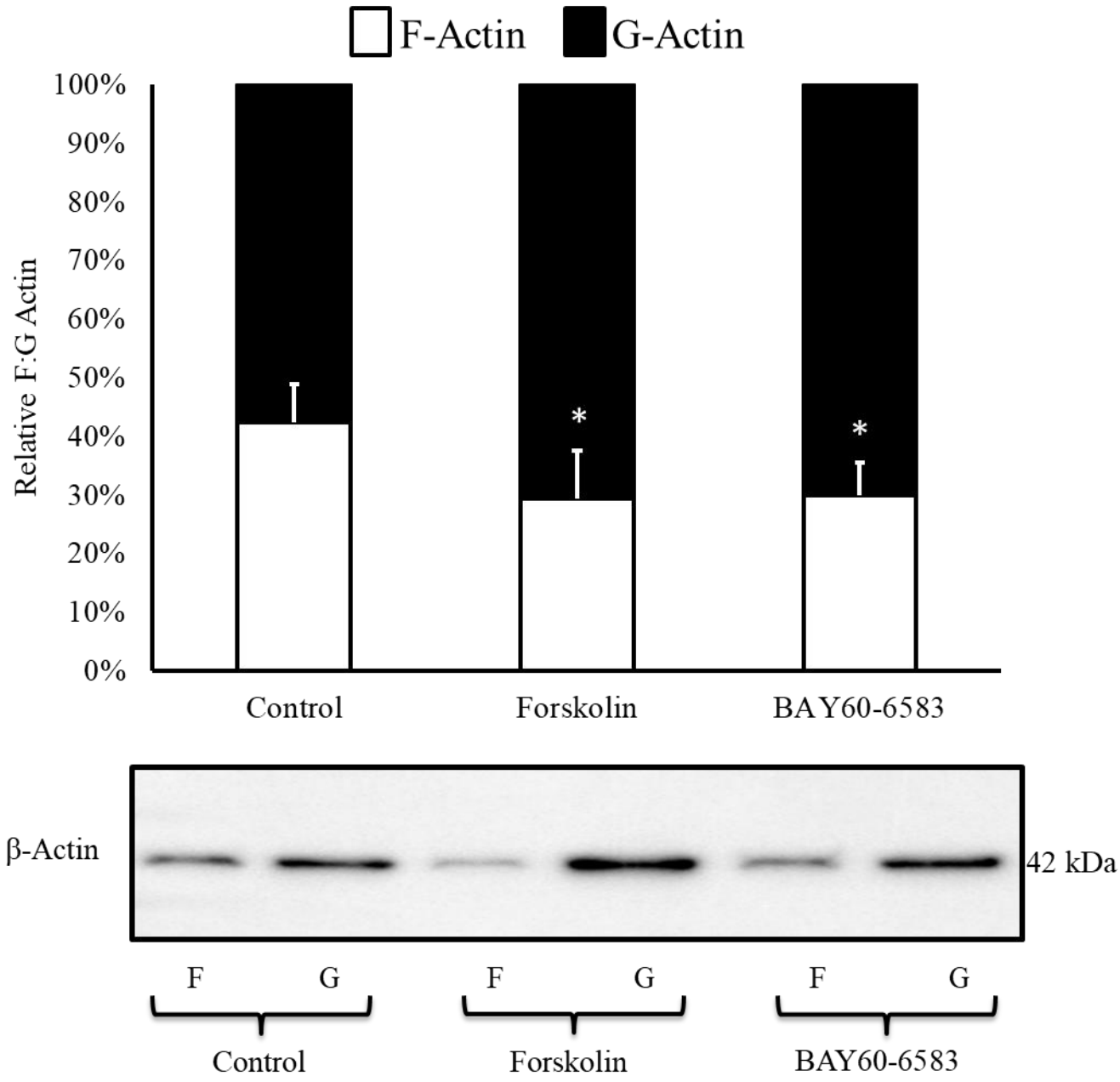


Figure 3.12: Elevated cAMP decreases F-actin fibres but increases G-actin fibres in cardiac fibroblasts

Rat cardiac fibroblasts were serum-starved for 4 hours before stimulation with 25 µM forskolin or 5 µg/ml BAY60-6583 in serum-free conditions for the indicated times. F-actin fibres and G-actin fibres were quantified by western blotting. *: $p<0.05$; F: F-actin and G: G-actin. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

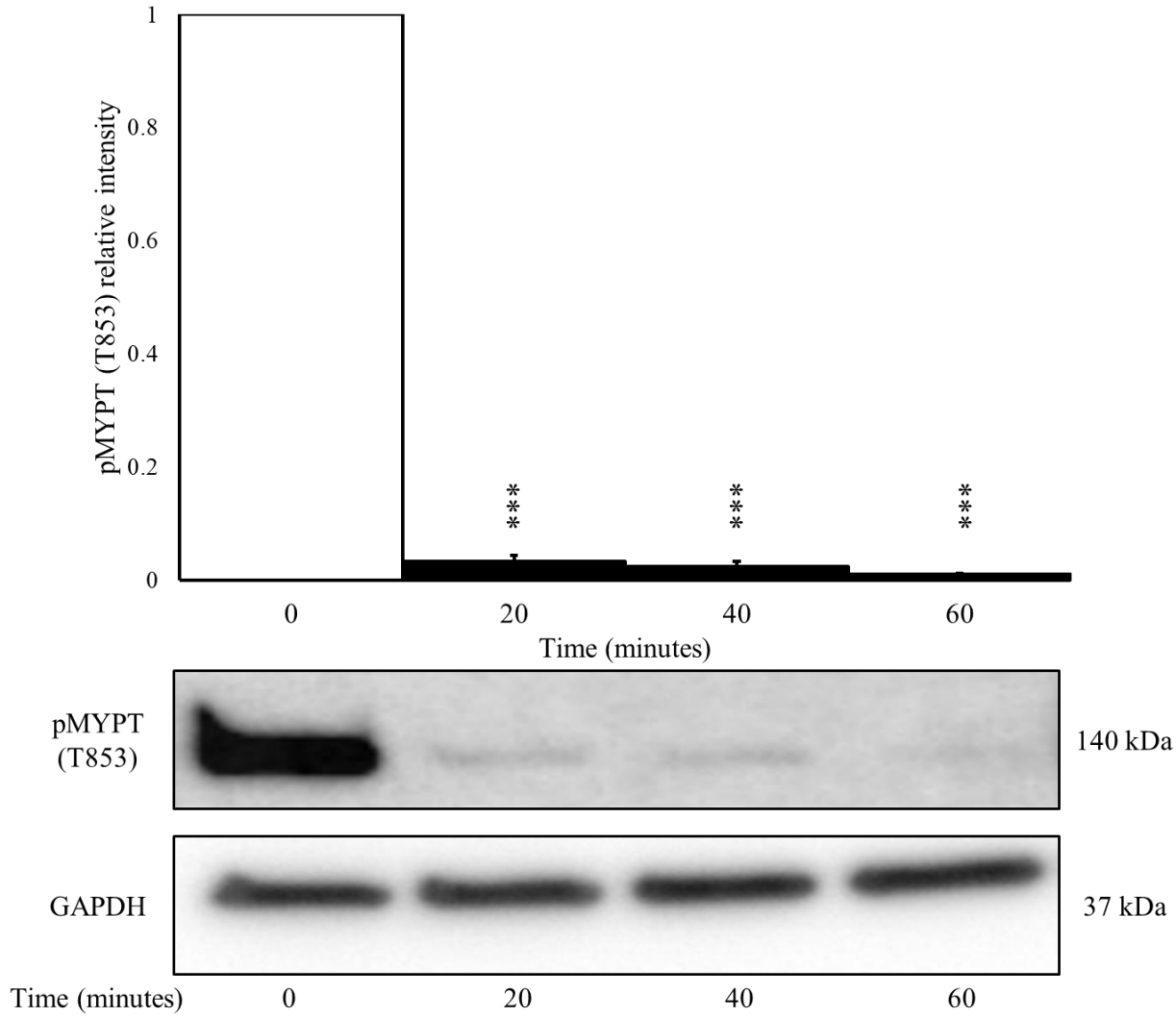


***Figure 3.13: Y27632* suppresses phosphorylation of MYPT in cardiac fibroblasts.**

Rat cardiac fibroblasts were treated with 10 μM Y27632 for indicated times and total cell lysates analysed for phosphorylated MYPT and GAPDH by western blotting. ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

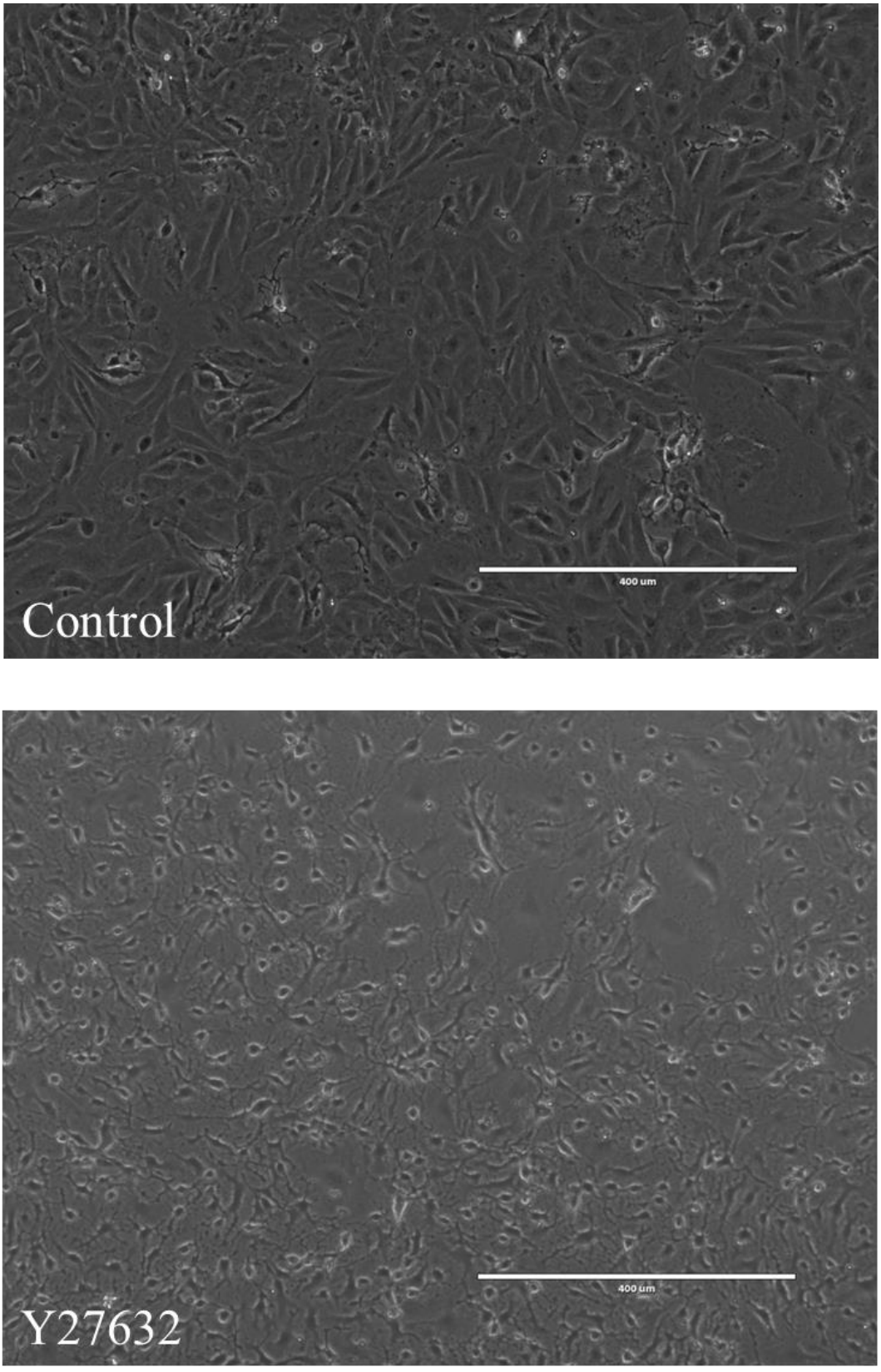


Figure 3.14: Y27632 induces morphological changes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated in serum-free conditions with 10 µM Y27632 and phase contrast images taken after 8 hours.

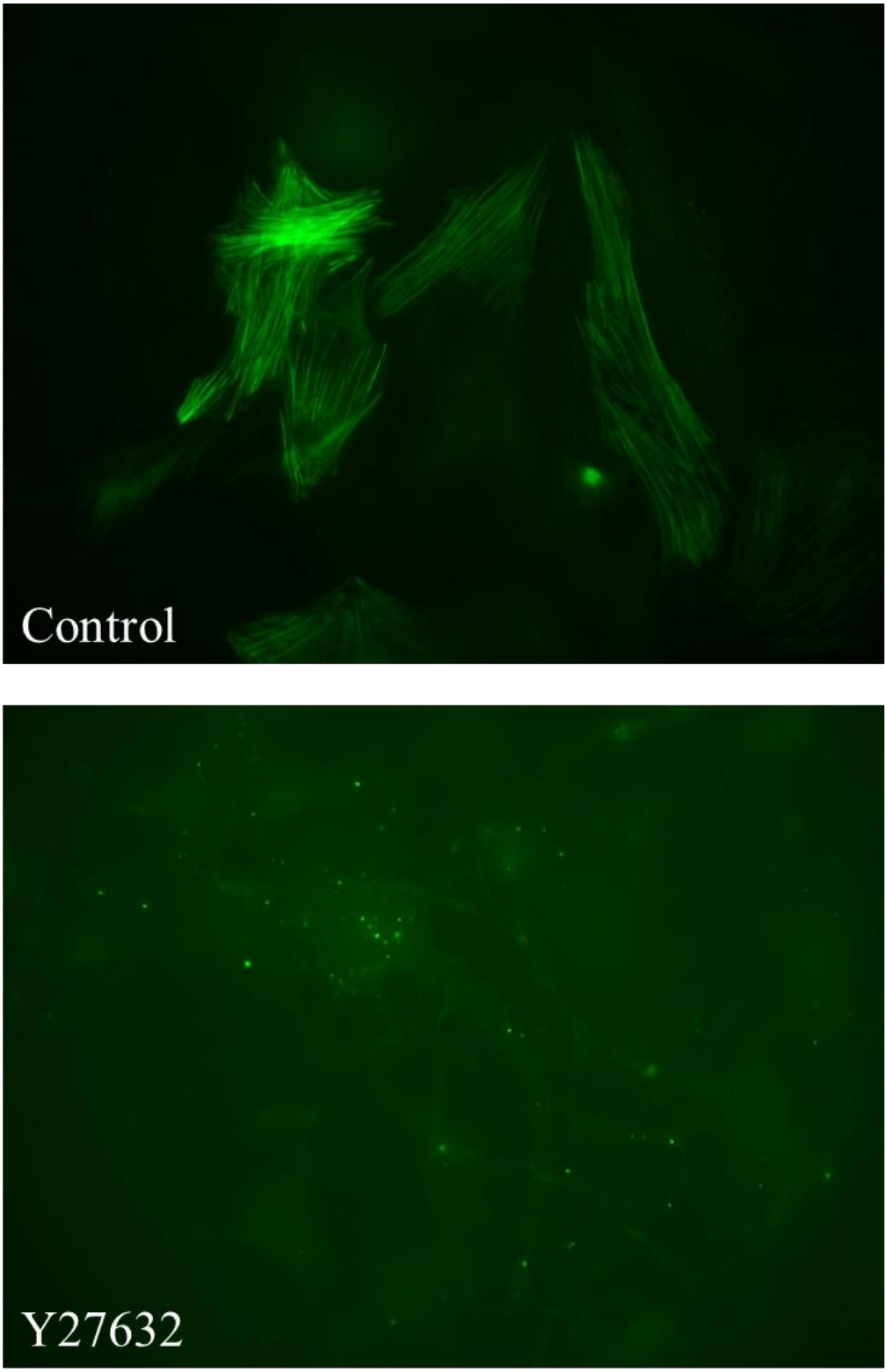


Figure 3.15: Y27632 disrupts the F-actin fibres in cardiac fibroblasts.

Rat cardiac fibroblasts were serum-starved for 4 hours before stimulation with 10 μM Y27632 in serum free conditions for indicated times. Polymerised F-actin filaments were detected using Alexa-fluor-488 phalloidin staining of fixed cells. Representative images are shown.

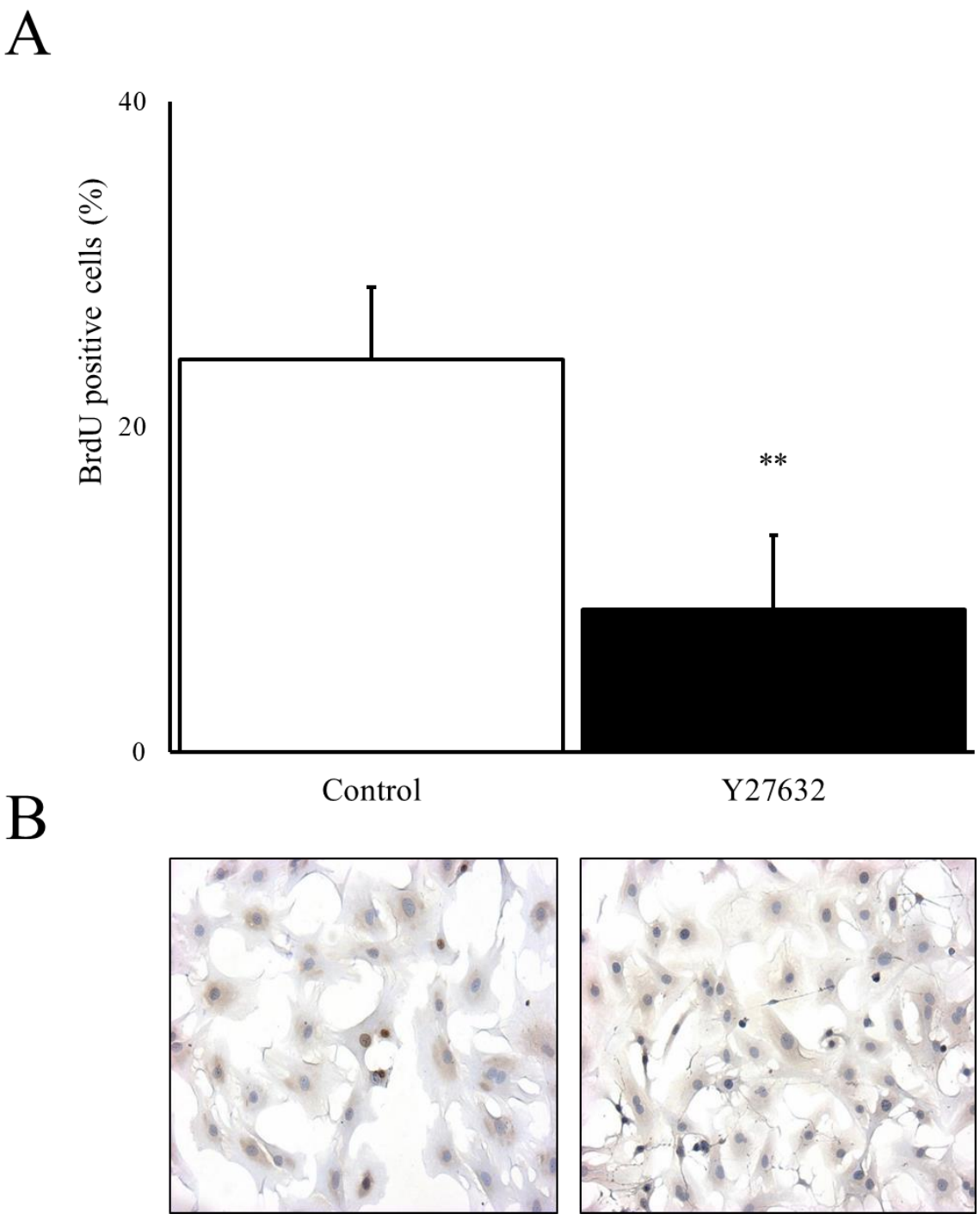


Figure 3.16: Y27632 inhibits serum-stimulated proliferation of rat cardiac fibroblasts

Rat cardiac fibroblasts were incubated for 18 hours with 10 µM Y27632 in 5% (v/v) serum. Cells were labelled with 10 µM BrdU for further 6 hours and proliferation quantified by immuno-histochemical staining of incorporated BrdU (**A**). Representative images of each condition are shown below (**B**). **: $p<0.01$ and Y27632: ROCK inhibitor. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

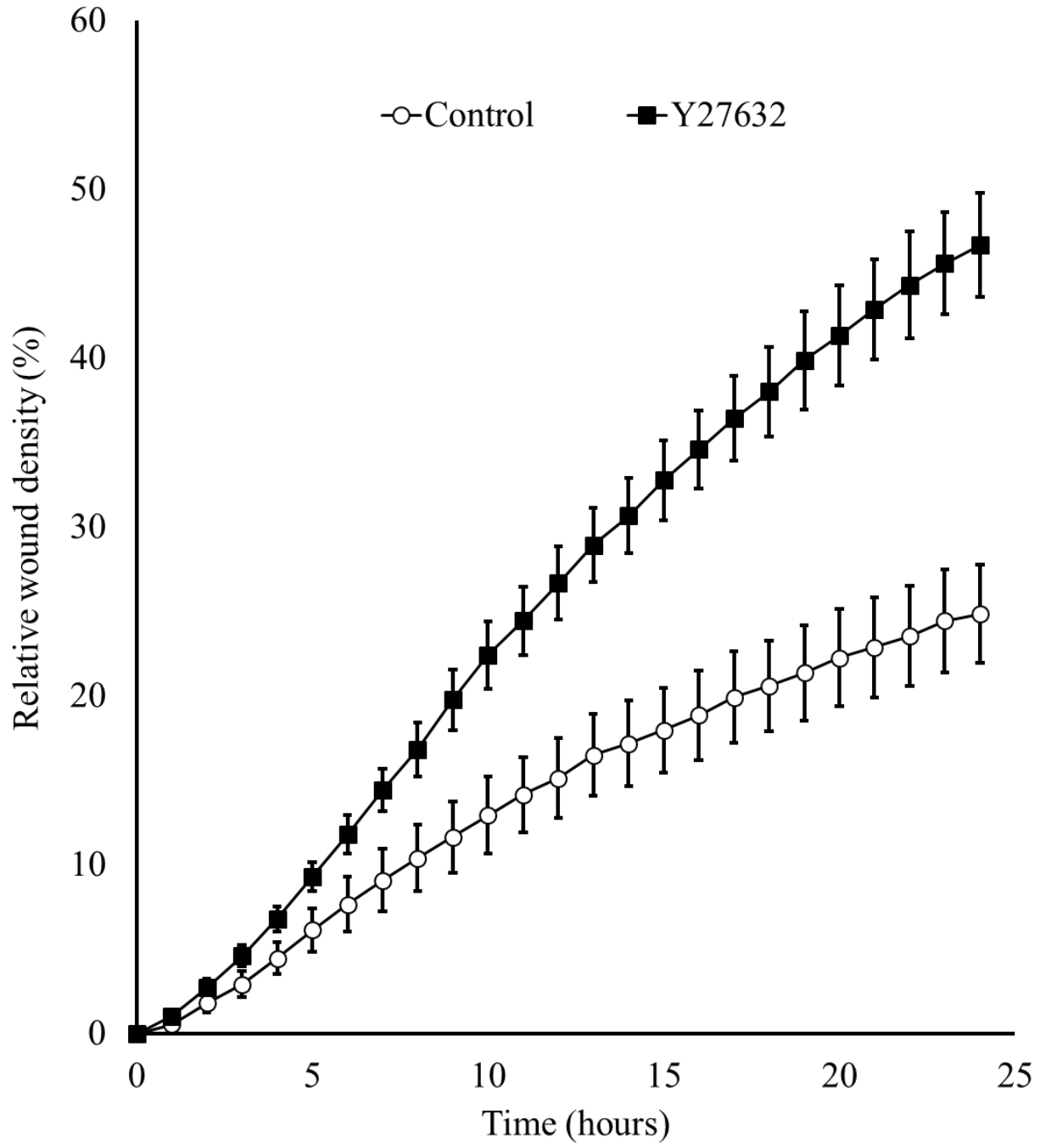


Figure 3.17: Y27632 increases the migration rates of cardiac fibroblasts.

Real-time scratch-wound migration analysis of rat cardiac fibroblasts stimulated with 10 µM Y27632 in 5% (v/v) FCS was performed using an IncuCyte ZOOM live-cell analysis system. Y27632: ROCK inhibitor. n=6.

3.4 DISCUSSION

In this chapter, the anti-proliferative and anti-migratory effects of elevated cAMP in cardiac fibroblasts were investigated. The data presented demonstrates that elevated cAMP, in response to activation of physiologically relevant GPCR receptors, such as the A2BR, or directly with synthetic cAMP analogues or the adenylyl cyclase activator forskolin, inhibits the proliferation but not the migration of rat cardiac fibroblasts. Using cAMP analogues to selectively activate either PKA or EPAC, it was demonstrated that both PKA and EPAC signalling have anti-mitogenic effects in these cells and together they act additively to inhibit cardiac fibroblast proliferation. Data is also presented demonstrating that the anti-mitogenic effects of cAMP in cardiac fibroblasts is associated with a dramatic remodelling of the actin cytoskeleton, characterised by loss of phalloidin stainable F-actin stress fibres, a decrease in the F-actin:G-actin ratio and a clear change in cell morphology that is similar to the stellate morphology reported in other cell types (Pelletier, Julien et al. 2005, Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011). This actin remodelling and morphological change requires coordinated signalling through both PKA and EPAC.

The data presented here demonstrate a role for adenosine signalling via the A2BR in inhibiting cardiac fibroblast proliferation. Extracellular nucleotides and nucleosides, such as adenosine, have been implicated as important regulators of cardiac physiology and cardiac fibroblasts homeostasis, including vasodilatation of coronary vessels, regulation of heart rate, angiogenesis, myocardial hypertrophy as well as modulation of cardiac fibrosis and regulation of cardiac fibroblast proliferation (Dubey, Gillespie et al. 1997, Dubey, Gillespie et al. 2001, Vecchio, White et al. 2017). Due to their central role in the cardiac fibrotic process, increased cardiac fibroblast proliferation is likely be an important factor in promoting cardiac fibrosis. Adenosine has been described as a ‘retaliatory metabolite’ due to its enhanced release and production, and ability to help restore energy balance during times of cellular metabolic stress or injury (Newby 1984, Shryock and Belardinelli 1997). Extracellular adenosine levels can be increased in response to tissue damage, ischemia or a crisis event (Hori and Kitakaze 1991, Mubagwa, Mullane et al. 1996). Damage to the cell membranes can cause release of ATP into the extracellular space which then can be metabolised to adenosine via the action of ecto-5’-nucleotidase (Fredholm 2007, Hasko, Linden et al. 2008, Ohta and Sitkovsky 2014, Fan and Guan 2016). The cardio protective properties of adenosine in the heart has led to several clinical trials for adenosine-based therapies for the treatment of ischaemia reperfusion injury after MI (Kopecky, Aviles et al. 2003, Ross, Gibbons et al. 2005, White, Atrasz et al. 2005, Forman, Stone et al. 2006, Vecchio, White et al. 2017). The diverse functions of adenosine are mediated via the four main adenosine receptors, A1, A2A, A2B and A3, which are

all expressed in cardiac fibroblasts, with A2B receptors being the most abundant (Villarreal, Epperson et al. 2009). Here we demonstrated that activation of the A2BR using the selective A2BR agonist, BAY60-6583, inhibits cardiac fibroblast proliferation. This data is consistent with previous work implicating signalling through the A2BR in limiting cardiac fibrosis (Dubey, Gillespie et al. 1998, Dubey, Gillespie et al. 2001, Villarreal, Epperson et al. 2009). In a rat model of MI, agents that elevate adenosine levels reduced tissue scar formation via the Gs-coupled A2BR and increased intracellular cAMP levels (Ryzhov, Sung et al. 2014). Consistent with these findings, silencing of the A2BR increases cardiac fibroblast proliferation and collagen synthesis whereas its overexpression has the opposite effects (Dubey, Gillespie et al. 2001, Chen, Epperson et al. 2004). Several lines of evidence suggest that these protective effects of adenosine are mediated by increases in intracellular cAMP. For example, over expression of adenylate cyclase or its activation with forskolin inhibits TGF-β or angiotensin II induced myofibroblast differentiation, collagen synthesis and proliferation in cardiac fibroblasts (Dubey, Gillespie et al. 2001, Swaney, Roth et al. 2005, Giannotta, Benedetti et al. 2014). Consistent with these reports, data presented in this chapter confirms that cAMP elevation in response to forskolin or Db-cAMP analogues stimulation significantly inhibited cardiac fibroblast proliferation.

Anti-proliferative properties of elevated cAMP have previously been reported in other mesenchymal cell types, including vascular smooth muscle cells (Hayashi, Morishita et al. 2000, Growcott, Spink et al. 2006, Smith, Hudson et al. 2017, Smith, Sessions et al. 2019). In VSMC, cAMP inhibits entry into S-phase of the cell cycle via a mechanism that requires the activation of both PKA and EPAC effectors (Hewer, Sala-Newby et al. 2011). Using PKA and EPAC selective cAMP analogues, work presented in this chapter demonstrates a similar requirement for PKA and EPAC signalling in cardiac fibroblasts. However, some key differences are evident. In VSMC, activation of PKA or EPAC in isolation did not significantly inhibit serum induced proliferation (Hewer, Sala-Newby et al. 2011). This contrasts with data presented here, which demonstrates a significant but submaximal inhibition of cardiac fibroblast proliferation in response to PKA or EPAC activation alone. In VSMC, PKA and EPAC activation acted synergistically to inhibit proliferation (Hewer, Sala-Newby et al. 2011), whereas in cardiac fibroblasts the effects were additive. The data presented here is consistent with data indicating an anti-mitogenic effect of selective EPAC or PKA activation (Yokoyama, Minamisawa et al. 2008). The reasons for these differences are not fully understood but may reflect differences in the relative expression levels of PKA and/or EPAC1 in these cell types or a difference in how these two cAMP effectors modulate downstream signalling pathways, including the actin remodelling mechanisms that have been

implicated in mediating cAMP-dependent anti-mitogenesis in VSMC (Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011). cAMP and EPAC signalling are known to have divergent effects on the proliferation in various cell types. For example, elevated cAMP inhibits proliferation of several cell types of mesenchymal origin, including VSMC (Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014, Smith, Hudson et al. 2017) and cardiac fibroblasts, but stimulates the proliferation of other cell types, including epithelial cells (Yamaguchi, Nagao et al. 2003), embryonic stem cells (Lee, Kim et al. 2012), endothelial cells (Smith, Hudson et al. 2017) and even VSMC under some conditions (Hogarth, Sandbo et al. 2004). Interestingly, the balance of PKA and EPAC signalling has been reported to switch cAMP signalling from a pro-mitogenic signal into an anti-mitogenic signal in PC12 cells, suggesting that the interplay between these two pathways is a critical step in determining the proliferative response (Kiermayer, Biondi et al. 2005).

The anti-mitogenic effects of cAMP-elevating stimuli in cardiac fibroblasts reported here were associated with a rapid and profound change cell morphology and disruption of the actin cytoskeleton. Cyclic-AMP elevating stimuli resulted in a loss of phalloidin stainable actin stress fibres (F-actin) and a significant reduction in the F-actin:G-actin ratio. Similar cAMP-induced morphological changes, described as a 'stellate morphology' and characterised by a condensation of the cell body, formation of long branching membrane processes and loss of actin stress fibres have been reporter previously in other cell types, including VSMC and astrocytes (Pelletier, Julien et al. 2005, Hewer, Sala-Newby et al. 2011, Chen, Wild et al. 2014, Smith, Hudson et al. 2017). These morphological changes and loss of actin stress fibres occur rapidly after cAMP elevation suggesting that they are an early event in the cAMP signalling mechanisms preceding changes in cell proliferation and occuring independently of changes in gene expression. Cell morphology and the integrity and the organisation of the actin cytoskeleton is known to be a critical permissive factor in cell-cycle progression of adhesion-dependent cell types. This is clearly demonstrated by numerous studies using actin binding drugs such as latrunculin-B or cytochalasin-D, which inhibit actin polymerisation and block progression past the G_1 phase restriction point that controls entry into S-phase of the cell cycle (Huang, Chen et al. 1998, Wang, Li et al. 2019). The implication is that cAMP-induced morphological changes and inhibition of actin polymerisation are likely to contribute, at least in part, to the anti-mitogenic effects of cAMP in cardiac fibroblasts. The mechanisms underlying this are investigated in more detail in chapter 4. Consistent with a requirement for actin polymerisation for a pro-fibrotic cardiac fibroblast phenotype, a recent study reported that fibroblasts isolated from rat hearts after 4 weeks of volume overload induced by experimental aortocaval fistula displayed a hypo-fibrotic phenotype that was associated with a 78%

reduction in F-actin and a reduced F-actin:G-actin ratio, compared to controls (Childers, Sunyecz et al. 2019). Importantly, treatment with a ROCK inhibitor (Y27632) reduced the F-actin:G-actin ratio and replicated the hypo-fibrotic phenotype. Moreover, promoting actin polymerisation with the actin binding drug jasplakinolide, increased the F-actin:G-actin ratio and restored a more fibrotic response in the cells from the volume overload hearts (Childers, Sunyecz et al. 2019). Taken together, these observations suggest that cAMP-induced remodelling of the actin cytoskeleton plays an important role in regulating a pro-fibrotic and proliferative cardiac fibroblast phenotype. The data presented in this chapter demonstrates that elevation of cAMP using either BAY60-6583, or directly with forskolin, induced disruption of actin polymerisation. A similar morphological change and actin disruption was observed in response to selective PKA activation with 6-BNZ-cAMP-AM, which also inhibited proliferation. Similar PKA-induced changes in morphology, actin cytoskeleton remodelling and growth arrest have been reported previously in vascular smooth muscle cells (Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014). However, no morphological change or disruption of actin stress fibres in cardiac fibroblasts was detected in response to selective EPAC activation, using either 8-pCPT-cAMP-AM or a non-cyclic nucleotide EPAC agonist I-942, despite selective EPAC activation resulting in a significant, albeit submaximal, inhibition of proliferation. This lack of EPAC-dependent morphological or actin change is similar to effects reported in VSMC, although in VSMC, selective EPAC activation does not result in growth arrest, in contrast to the growth inhibitory effects in cardiac fibroblasts reported here. This implies that EPAC-mediated growth arrest in cardiac fibroblasts occurs independently of actin cytoskeleton changes. This may reflect activation of actin-independent EPAC-effector signalling pathways that contribute towards the growth inhibitor properties of cAMP in cardiac fibroblasts. For example, EPAC activates RAP1 (Chapter 5), independently of changes in actin remodelling. Importantly, the Rap1 gene was originally identified by its ability to revert Ras-mediated cellular transformation (Kitayama, Sugimoto et al. 1989), suggesting that EPAC-Rap1 signalling may underly the actin-independent growth inhibitory effects of EPAC signalling in cardiac fibroblasts. However, EPAC activation also enhances PKA-mediated morphological change, actin disruption and growth arrest, implying that at least some of the effects of EPAC signalling may be mediated via enhancement of PKA-induced actin remodelling.

Although there are several similarities between the effects of cAMP signalling reported here in cardiac fibroblasts and those reported previously in vascular smooth muscle cells, a major difference is the effects of cAMP elevation on migration in these two cell types. In vascular smooth muscle cells, cAMP elevating stimuli inhibit cell migration (Yokoyama, Minamisawa et al. 2008, Hayashi,

Murai et al. 2015, McKean, Murray et al. 2015, Smith, Hudson et al. 2017). The data presented here demonstrates that cAMP elevation in cardiac fibroblasts using forskolin or Db-cAMP analogue has the opposite effect, increasing the migration of cardiac fibroblasts. This implies that distinct signalling mechanisms are involved in the regulation of proliferation and migration in these cells.

Moreover, several lines of evidence reported the actin-sensitive transcription factor:co-factor complexes, MKL-SRF and YAP/TAZ-TEAD, to have crucial roles in the regulation of cellular proliferation and migration (Parmacek 2010, Yu and Guan 2013, Vasudevan and Soriano 2014, Duggirala, Kimura et al. 2015, Kimura, Duggirala et al. 2016, Joy, Gau et al. 2017, Smith, Hudson et al. 2017, Boopathy and Hong 2019). The regulation of these transcription factor complexes by cAMP in cardiac fibroblasts is presented in chapters 4 and 5 of this thesis and demonstrates that both are negatively regulated by cAMP. This clearly implies that cardiac fibroblasts migration can occur independently of MKL-SRF and YAP/TAZ-TEAD activity, which contrasts with the role of the factors in vascular smooth muscle cells.

The importance of actin remodelling for cell migration and motility is widely accepted. Cell migration requires continuous dynamic reorganisation of actin, including increased actin polymerisation to promote lamellipodia formation and strengthen new focal adhesions at the leading edge but at the same time requires actin depolymerisation and focal adhesion disassembly at the rear, allowing for retraction of the trailing edge driven by actomyosin contractility (Pollard and Cooper 2009). Rho-GTPase activity plays a key role in promoting actin polymerisation at the leading edge and is essential for migration of cardiac fibroblasts in two-dimensional migration assays . Silencing of RhoA in cardiac fibroblasts inhibits their migration in 2D scratch wound assay (Jatho, Hartmann et al. 2015), consistent with a requirement for RhoA-mediated actin polymerisation. However, silencing of RhoA enhances the migration of cardiac fibroblast through a three-dimensional collagen gel by allowing and promoting an amoeboid mode of migration (Jatho, Hartmann et al. 2015). This suggests that cardiac fibroblasts use two mechanistically distinct modes of migration, a RhoA and actin-dependent mesenchymal mode of migration and a RhoA and actin independent amoeboid mode of migration. The data presented here demonstrates that cAMP elevation with forskolin, Db-cAMP analogue or Y27632 does not inhibit cardiac fibroblast migration despite clear inhibitory effects on actin polymerisation. This may indicate that cardiac fibroblasts adopt an amoeboid mode of migration under conditions of elevated cAMP and impaired actin polymerisation. Yokoyama et al also reported enhanced cardiac fibroblast migration in response to elevated cAMP. However, this enhancement was only evident with low but not high levels of cAMP (Yokoyama, Patel et al. 2008). The inhibitory effects of higher concentrations of

forskolin reported by Yokoyama may be due to migration assays being performed under serum free conditions, which contrasts with the migration assay data presented in this chapter. Serum mitogens activate Rho-GTPase activity (Toksoz and Merdek 2002, Maddala, Reddy et al. 2003, Ravi, Kaushik et al. 2015), suggesting that these differences may reflect the magnitude of inhibition of Rho-GTPase activity and actin polymerisation. In this chapter we present data quantifying the reduction in the F-actin:G-actin ratio in response to forskolin. These data demonstrate that cAMP elevation reduces the percentage of F-actin from 40% to 30%. Importantly, similar analyses in vascular smooth muscle cells demonstrate a much larger reduction in F-actin content (from 57% to 9%). This larger inhibition of actin polymerisation by cAMP in VSMC may explain the different effects of cAMP in vascular smooth muscle cells and cardiac fibroblasts. The physiological significance of the divergent effects of cAMP on cardiac fibroblast migration is unknown. However, the ability of cardiac fibroblast to migrate under conditions of elevated cAMP and impaired actin polymerisation, possibly by adopting an amoeboid mode of migration, may reflect that critical importance of rapid cardiac repair following acute myocardial injury, such as following MI.

Furthermore, we also showed that cAMP elevating stimuli induce significant morphological changes, actin depolymerisation and downregulation of proliferation of cardiac fibroblasts, but a small increase in their migration. Previous investigations have linked cAMP signalling with inhibition of the RhoA-ROCK pathway (Gao, Li et al. 2004). Also our group suggested that, the effects induced by cAMP in vascular smooth muscle cells is due to its inhibitory effects on Rho/ROCK signalling (Smith, Hudson et al. 2017). In order to test the hypothesis that cAMP-mediated inhibition of ROCK may contribute towards the inhibitory effects of cAMP on cardiac fibroblast proliferation, cells were treated with the ROCK inhibitor Y27632. This effectively inhibited ROCK activity indicated by a large reduction in phosphorylation of the ROCK substrate MYPT. Importantly, pharmacological ROCK inhibition replicated the effects of elevated cAMP, inducing a stellate morphology, loss of actin stress fibres and inhibition of cell proliferation, without inhibiting cell migration. In fact, ROCK inhibition actually stimulated migration of cardiac fibroblasts in the experiments presented here. The role of ROCK activity on cell migration is controversial. Several lines of evidence have demonstrated that ROCK inhibition reduces cell migration (Sadok, McCarthy et al. 2015, Wang, Yang et al. 2016, Guerra, Oliveira et al. 2017, Smith, Hudson et al. 2017). However, other studies indicate that ROCK signalling represses migration (Totsukawa, Wu et al. 2004, Yang and Kim 2014, Piltti, Varjosalo et al. 2015, Chang, Zhang et al. 2018). This may reflect cell type specific differences. Stimulation of cardiac fibroblasts with Y27632 increased the migration of cardiac fibroblasts in real-time scratch wound assays.

Again, this could imply that cardiac fibroblasts adopt an amoeboid mode of migration under conditions when ROCK is inhibited, or their actin filaments are depolymerised.

In summary, data presented in this chapter demonstrates that the physiological GPCR agonist adenosine and elevated levels of cAMP inhibits cardiac fibroblast proliferation but not migration and that this inhibitory action is associated with a rapid change in cell morphology and inhibition of actin polymerisation. These data suggest that increases in adenosine following cardiac injury may play an important role in retraining cardiac fibroblast proliferation and fibrosis following cardiac injury. The proliferative expansion of the cardiac fibroblast population, the main extracellular producing cell type in the myocardium, enhances the deposition of extracellular proteins and hence contributes to fibrosis. Therefore, limiting cardiac fibroblast proliferation may be a physiological response to prevent excessive fibrosis and may represent a potential therapeutic avenue to reduce pathological myocardial fibrosis. The data of this chapter is summarised in figure 3.18.

The underlying signalling mechanisms mediating these anti-mitogenic properties of cAMP will be investigated in chapter 4

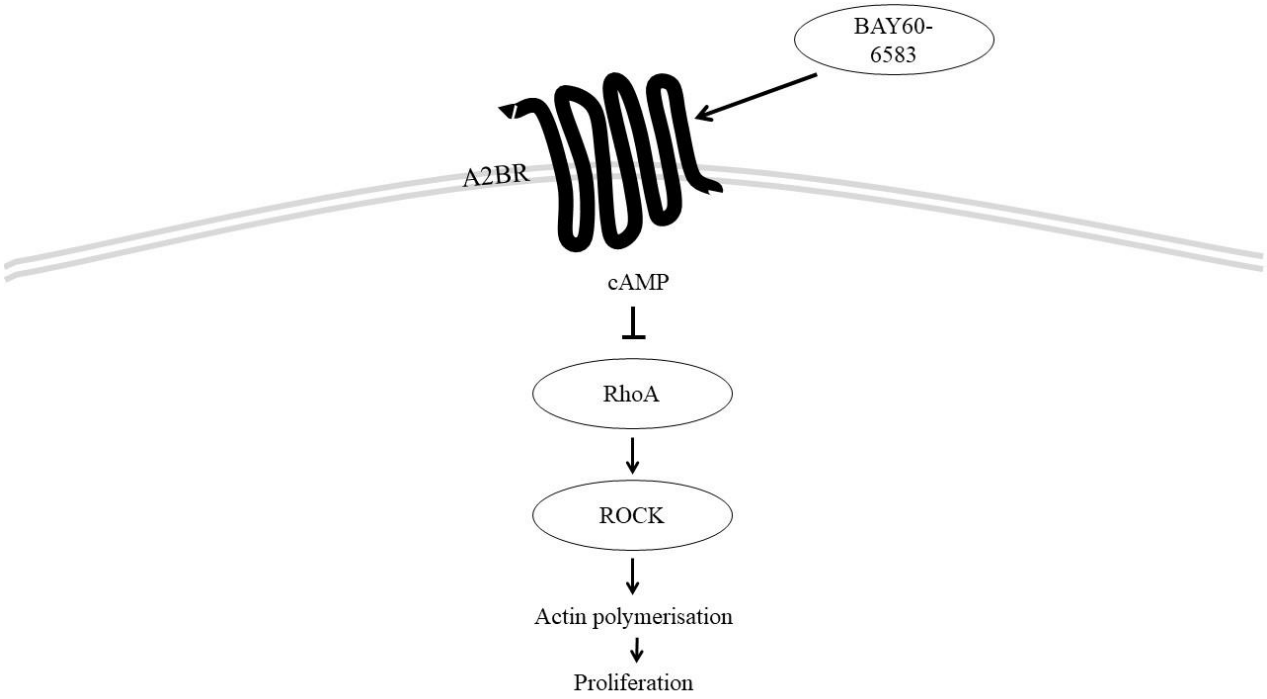


Figure 3.18. Schematic representation of regulation cardiac fibroblast proliferation by adenosine induced cAMP signalling.

Adenosine signalling via the adenosine A2B receptor induces cAMP levels in cardiac fibroblast. This inhibits RhoA-ROCK signalling and actin polymerisation, resulting in a change in cell morphology and inhibition of cell proliferation.

CHAPTER 4:

ROLE OF MKL1-SRF AND YAP/TAZ-TEAD IN THE ANTI-MITOGENIC EFFECTS OF cAMP IN CARDIAC FIBROBLASTS

4.1 INTRODUCTION

In chapter 3, data was presented demonstrating that cAMP-elevating stimuli inhibit cardiac fibroblast proliferation. This anti-mitogenic effect was associated with a rapid and dramatic change in cell morphology and a reduction in actin polymerisation. However, it was not clear if these anti-mitogenic effects were mediated by the associated changes in actin polymerisation. Furthermore, it was not determined what the underlying mechanisms might be that link cAMP-dependent actin remodelling to changes in cardiac fibroblast proliferation.

As discussed in chapter 3, morphological changes and loss of actin stress fibres occur rapidly after cAMP elevation, suggesting that they are an early event in the cAMP signalling mechanisms and precede changes in cell proliferation. The rapid onset of these morphological and actin changes also imply that they probably occur independently of changes in gene expression. Cell morphology, cell spreading and the polymerisation of the actin cytoskeleton are known to be critical factors in allowing cell proliferation of many adhesion-dependent cell types (Smith, Sessions et al. 2019). Studies using actin binding drugs such as cytochalasin-D or latrunculin-B, which directly inhibit actin polymerisation clearly demonstrate that the integrity of the actin cytoskeleton is essential for cell cycle progression in many different adhesion-dependent cell types (Huang, Chen et al. 1998, Wang, Li et al. 2019). Several mechanisms have been proposed to be involved in mediating the actin-dependence of cell proliferation. Early studies highlighted effects on the MAPK signalling pathway (Aplin, Howe et al. 1999, Schwartz and Assoian 2001). However, several lines of evidence reported that the ability of a polymerised actin cytoskeleton to support cell proliferation is not mediated simply via the MAPK signalling pathway. In fact, MAPK signalling is only important in the early G_1 phase of the cell-cycle while a properly organised actin cytoskeleton is essential throughout most of the G_1 phase up until the restriction point that controls entry into S-phase (Wang, Li et al. 2019). These studies imply that effects of the actin cytoskeleton on proliferation can be separated from actin-dependent effects on MAPK activity. Interestingly, the anti-mitogenic effects of cAMP signalling on cell proliferation have also been shown to be dissociated from the inhibitory effects of cAMP on MAPK signalling (Cospedal, Lobo et al. 1999). These data imply that additional mechanisms are involved in mediating the anti-mitogenic effects of cAMP and actin disruption. Early work from our lab implicated remodelling of the actin cytoskeleton as playing an important functional role in mediating the anti-mitogenic effects of cAMP in VSMC (Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014). Consistent with this, data presented in chapter 3 demonstrates that cAMP is also anti-mitogenic in cardiac fibroblasts and that this is also associated with actin remodelling and pronounced morphological changes. The fundamental

mechanisms involving actin cytoskeleton remodelling and changes in gene expression are therefore likely to be important in allowing cells to sense and respond to cAMP-elevating stimuli, in addition to various other micro-environmental signals that regulate the polymerisation of actin cytoskeleton. A large body of research has focussed on characterising these mechanisms. Early research studying the regulation of the *FOS* gene helped to identify a key regulatory mechanism that links the actin-cytoskeleton to changes in gene expression (Graham and Gilman 1991, Hill, Wynne et al. 1995). The *FOS* gene is controlled by binding of the SRF transcription factor to a serum-response element (SRE) in its proximal promoter region. Mitogen stimulation was shown to be able to induce the *FOS* gene, via activation of the MAPK signalling pathway, by inducing the binding of ternary complex factor (TCF) binding to SRF. However, mutated SRF elements in the *FOS* promoter that were incapable of binding TCFs remained responsive to signals that modulated Rho-GTPase activity or induced remodelling of the actin cytoskeleton (Graham and Gilman 1991, Hill, Wynne et al. 1995). This work ultimately established that SRF activity is also sensitive to actin polymerisation via a TCF-independent mechanism (Posern, Miralles et al. 2004). As mentioned in chapter 1, regulation of SRF activity following actin remodelling is regulated by SRF co-transcription factors, known as MKL1 and MKL2 (Miralles, Posern et al. 2003). As discussed in the introduction chapter, MKL1 and MKL2 translocate between the cytosol and the nucleus, where they interact with and stimulate SRF-dependent gene expression (Miralles, Posern et al. 2003). The cellular localisation of MKL proteins is regulated by interaction with G-actin monomers, which bind to MKL1/2 via their N-terminal RPEL domains. It follows that signals that reduce actin polymerisation, such as cAMP-elevating stimuli, increase the association of actin monomer with the MKL1/2. Inhibition of actin polymerisation using actin binding drugs has been shown to induce nuclear export of MKL1/2 and hence reduced SRF-dependent gene expression (Esnault, Stewart et al. 2014). In contrast, stimuli such as mitogens that increased RhoA-mediated actin-polymerisation and hence depletion of G-actin monomer levels cause MKL1/2 to translocate into the nucleus, where they enhances SRF-dependent gene expression (Guettler, Vartiainen et al. 2008, Hirano and Matsuura 2011, Smith, Hudson et al. 2017). Through investigating the effects of cAMP stimulation on actin cytoskeleton remodelling in cardiac fibroblasts (which was discussed in chapter 3), it became a point of interest to study whether MKL1 and MKL2 are also regulated by cAMP in cardiac fibroblasts and if this mechanism is involved in mediating the anti-mitogenic effects of cAMP in these cells. Our group has previously reported that RhoA inhibition downregulates the expression of SRF-dependent genes in VSMCs (Duggirala, Kimura et al. 2015). Furthermore, forskolin stimulation was demonstrated to inhibit the expression of pro-proliferative and pro-migratory genes: *Ccn1* and *Ctgf*, via a pathway dependent on MKL1 (Duggirala, Kimura et al. 2015, Kimura,

Duggirala et al. 2016, Smith, Hudson et al. 2017). These results indicate that cAMP signalling represses the expression of SRF-dependent genes by regulating MKL1 activation. This mechanism may contribute towards the anti-mitotic effects of cAMP in fibroblasts, given that pharmacological inhibition or genetic deletion of MKL1/2 reduced intestinal fibrosis (Johnson, Rodansky et al. 2014), lung fibrosis (Sisson, Ajayi et al. 2015), skin fibrosis (Haak, Tsou et al. 2014) and inhibited the expression of ECM genes and smooth muscle genes associated with a myofibroblast phenotype (Small, Thatcher et al. 2010).

Clearly, the effects of cAMP stimulation and actin depolymerisation are unlikely to be mediated solely via the MKL-SRF signalling pathway. Other actin sensitive transcription factors have also been described (Low, Pan et al. 2014, Meng, Moroishi et al. 2016, Qiao, Chen et al. 2017). For example, the transcriptional co-activators YAP and TAZ have also been implicated in linking actin remodelling and biomechanical signals to changes in gene expression and cellular responses in various cell types (Low, Pan et al. 2014, Park, Shin et al. 2018). These co-factors translocate between the cytoplasm and the nucleus in response to bio-mechanincal signals and stimuli that modulate actin organisation (Elosegui-Artola, Andreu et al. 2017, Dobrokhotov, Samsonov et al. 2018, Shreberk-Shaked and Oren 2019). Once in the nucleus, YAP and TAZ bind to TEAD transcription factors (TEAD1-4) and interact with other members of the transcriptional machinery, including RNA polymerase II (Rognoni and Walko 2019), to mediate the expression of different genes required for proliferation and migration (Komuro and Yacubova 2003, Zhao, Li et al. 2010, Hudson, Kimura et al. 2018). Under normal conditions, YAP and TAZ are negatively regulated by MST and LATS (Dong, Feldmann et al. 2007, Hao, Chun et al. 2008). Once YAP and TAZ are phosphorylated by LATS, they are translocated out of the nucleus into the cytoplasm and get degraded, which decreases the expression of TEAD-dependent genes (Zhao, Wei et al. 2007, Gloerich and Bos 2010). Recent investigations demonstrated that the activities of YAP and TAZ can be affected by GPCR signalling. For example, lysophosphatidic acid (LPA) and sphingosine 1-phosphophate (S1P) act via $G_{12/13}$-coupled receptors to activate YAP and TAZ, whereas stimulation of Gs-coupled receptors in response to epinephrine or glucagon inhibits YAP and TAZ function (Yu, Zhao et al. 2012). Consistent with this, recent research in smooth muscle cells and breast cancer cells has demonstrated that stimulation of cAMP signalling, which inhibits Rho-GTPase activity and inhibits actin polymerisation (Pelletier, Julien et al. 2005, Bond, Wu et al. 2008), induces YAP/TAZ phosphorylation, YAP/TAZ cytoplasmic translocation and downregulation of TEAD-dependent gene expression (Bond, Wu et al. 2008, Yu, Zhang et al. 2013, Kimura, Duggirala et al. 2016). This inhibition of YAP/TAZ activity has been implicated in mediating the anti-mitogenic effects of cAMP in VSMC since YAP and TAZ silencing in these cells inhibited

their proliferation (Kimura, Duggirala et al. 2016, Wang, Li et al. 2019) and constitutively active mutants of YAP and TAZ are able to reverse these anti-mitogenic properties of elevated cAMP (Kimura, Duggirala et al. 2016). Together, these data highlight the importance of YAP and TAZ in promoting the proliferation of cardiovascular cells and that the inhibition of YAP and TAZ contributes to anti-proliferative effects of cAMP (Kimura, Duggirala et al. 2016). Several lines of evidence suggested that YAP-dependent activation of TEAD enhances cell proliferation by increasing the expression of proliferation associated genes, such as *Ccn1* and *Ctgf* (Zhao, Ye et al. 2008, Stein, Bardet et al. 2015, Kimura, Duggirala et al. 2016, Wang, Li et al. 2019). Importantly, pro-mitotic and pro-migratory genes, such as *Ccn1* and *Ctgf* are also subject to regulation by MKL1/2-SRF. This suggests that genes required for efficient proliferation of cardiac cells are coupled to the status of the actin network via both MKL1/2-SRF and YAP/TAZ-TEAD transcriptional complexes (Smith, Sessions et al. 2019). Taken together, these data suggest that changes in actin organisation regulates cellular responses, including proliferation and migration. This regulation of cellular behaviour is at least in part by controlling the activities of different transcription factor complexes, such as, MKL1-SRF and YAP/TAZ-TEAD (Miralles, Posern et al. 2003, Camoretti-Mercado, Dulin et al. 2005, Ota and Sasaki 2008, Gau, Veon et al. 2017, Tsai, Anderson et al. 2017). Modifications in the stimulation of these transcription factor complexes, ultimately induce changes in cellular responses by changing gene expression (Minami, Kuwahara et al. 2012, Haak, Tsou et al. 2014). Notably, MKL-SRF and YAP/TAZ/TEAD have also been implicated in enhancing in tissue fibrosis following injuries and insults. Data presented in chapter 3 of this thesis, presented that elevated levels of cAMP down regulated the proliferation of cardiac fibroblasts. An effect which is associated with a rapid actin cytoskeleton remodelling. The aims and hypothesis of this chapter are mentioned in the next section.

4.2 HYPOTHESIS

The hypotheses for this chapter were:

1. cAMP elevation reduces SRF and TEAD-dependent transcription in cardiac fibroblasts
2. cAMP elevation inhibits nuclear translocation of MKL1 in cardiac fibroblasts
3. The proliferation of cardiac fibroblasts is MKL-dependent
4. cAMP elevation inhibits nuclear translocation of YAP in cardiac fibroblasts
5. The proliferation of cardiac fibroblasts is YAP/TAZ-dependent

4.3 RESULTS

4.3.1 Elevated levels of cAMP inhibit SRF and TEAD-dependent luciferase reporter gene activity in cardiac fibroblasts

Cardiac fibroblasts were transiently transfected with luciferase reporter gene constructs under the control of promoter regions containing either five multimerised copies of a consensus SRF element (SRF-LUC) or eight multimerised copies of a consensus TEAD element (TEAD-LUC). These cells were then stimulated with the A2BR agonist BAY60-6583, forskolin or Db-cAMP analogue for eight hours to increase the intracellular concentrations of cAMP (Figure 4.1). Treatment of cardiac fibroblasts with BAY60-6583, forskolin or Db-cAMP analogue resulted in a significant down regulation (to 0.67±0.12-fold $p<0.05$, 0.3±0.035-fold $p<0.01$ and 0.60±0.13-fold $p<0.05$, respectively) of SRF-dependent luciferase reporter gene activity (Figure 4.1). In a similar manner, stimulation of cardiac fibroblasts with BAY60-6583, forskolin or Db-cAMP analogue resulted in a significant down regulation (to 0.44±0.056-fold $p<0.001$, 0.22±0.036-fold $p<0.001$ and 0.41±0.054-fold $p<0.001$, respectively) of TEAD-dependent luciferase reporter gene activity (Figure 4.1). In contrast, the activity of the control luciferase reporter gene (Control-LUC), which was under the control of a minimal promoter region lacking any SRF or TEAD binding elements, was not significantly changed with these cAMP elevating agents (Figure 4.1). These data demonstrate that elevated cAMP inhibits SRF and TEAD-dependent luciferase reporter gene activities in cardiac fibroblasts.

4.3.2 Elevated levels of cAMP down regulate the expression of SRF and TEAD-dependent target genes in cardiac fibroblasts

As discussed previously, cAMP elevation has previously been linked to decreases in SRF and TEAD-dependent gene expression in VSMC by inducing actin remodelling and inhibiting the nuclear translocation of SRF transcription factors, MKL1 and MKL2 (Kimura, Duggirala et al. 2016, Smith, Hudson et al. 2017, Smith, Sessions et al. 2019). Since cAMP elevating stimuli inhibited the SRF and TEAD-dependent luciferase reporter gene activity, we tested if the endogenous SRF and TEAD-dependent target genes were also repressed by these cAMP-elevating stimuli in cardiac fibroblasts, using qRT-PCR. Cardiac fibroblasts were stimulated with 5 µg/ml BAY60-6583 and mRNA expression of previously characterised (Smith, Hudson et al. 2017) SRF target genes, *Ccn1*, *Ctgf*, *Serpine* (*Pai1)*, *Zyx*, *Heyl* and *Thbs1* quantified. Stimulation with BAY60-6583 rapidly and significantly inhibited the mRNA levels of *Ccn1* (to 0.25±0.17-fold, $p<0.05$, *Ccn1* pre-spliced RNA (to 0.4±0.2-fold, $p<0.05$), *Ctgf* (to 0.25±0.17-fold, $p<0.05$), *Ctgf* pre-spliced RNA (to 0.16±0.08-fold, $p<0.05$), *Serpine/Pai1* (to 0.49±0.23-fold, $p<0.05$), *Zyx* (to 0.45±0.09-fold,

p<0.05), *Heyl* (to 0.63±0.067-fold, p<0.05) and *Thbs1* (to 0.17±0.085-fold, p<0.05) after 6 hours respectively, while the mRNA levels of the housekeeping gene 36B4 was not affected (Figure 4.2). Moreover, stimulation of cardiac fibroblasts with 25 μM forskolin rapidly and significantly inhibited the mRNA levels of *Ccn1* (to 0.092±0.05-fold and to 0.05±0.016-fold at 2 and 6 hours respectively, p<0.001), *Ccn1* pre-spliced RNA (to 0.069±0.01-fold and to 0.087±0.04-fold at 2 and 6 hours respectively, p<0.001), *Ctgf* (to 0.26±0.11-fold and to 0.03±0.011-fold at 2 and 6 hours respectively, p<0.001), *Ctgf* pre-spliced RNA (to 0.065±0.033-fold and to 0.074±0.025-fold at 2 and 6 hours respectively, p<0.001), *Serpine/Pai1* (to 0.28±0.036-fold and to 0.015±0.006-fold at 2 and 6 hours respectively, p<0.001), *Zyx* (to 0.57±0.1-fold and to 0.23±0.08-fold at 2 and 6 hours respectively, p<0.001), *Heyl* (to 0.74±0.095-fold and to 0.7±0.2-fold at 2 and 6 hours respectively, p<0.001) and *Thbs1* (to 0.65±0.03-fold and to 0.09±0.02-fold at 2 and 6 hours respectively, p<0.001), while the mRNA levels of *36B4* were not affected (Figure 4.3). Taken together, these data indicate that cAMP elevating stimuli inhibit the expression of SRF and TEAD-dependent gene expression in cardiac fibroblasts.

4.3.3 Elevated levels of cAMP activate CREB-dependent transcription in cardiac fibroblasts

The activity of CREB-dependent gene expression was also quantified in order to examine whether the inhibition of SRF and TEAD-target gene mRNA expression by cAMP elevating stimuli was simply due to a generalised global repression of transcription or if this represented a more selective repression of specific transcription factor activity.

Cardiac fibroblasts were transiently transfected with a luciferase reporter gene constructs (CREB-LUC) under the control of a 168 bp of the endogenous glycoprotein human alpha-subunit gene promoter, which contains two copies of a consensus CREB gene promoter. This vector was a gift from Professor Stan McKnight and has previously been shown to faithfully report CREB activity (Mellon, Clegg et al. 1989, Smith, Hudson et al. 2017). Cardiac fibroblasts were transfected with CREB-LUC and stimulated with BAY60-6583, forskolin or Db-cAMP and luciferase activity quantified. Treatment of cardiac fibroblasts with BAY60-6583, forskolin or Db-cAMP analogue resulted in a significant up regulation (to 8.09±1.40-fold, p<0.001; 4.30±1.16-fold, p<0.05 and 5.34±1.23-fold, p<0.05 respectively) of CREB-LUC (Figure 4.4). Furthermore, the mRNA expression levels of the endogenous CREB-target gene, *Nr4A1* (Volakakis, Kadkhodaei et al. 2010, Medzikovic, de Vries et al. 2019), was also significantly upregulated in response to BAY60-6583 (to 10.7±3.26-fold and 6.68±1.58-fold, p<0.05 at 2 and 6 hours of stimulation respectively, Figure 4.5A), forskolin (to 16.3±7.91-fold, p<0.05 and 2.20±0.91-fold at 2 and 6 hours of stimulation respectively, Figure 4.5B) or Db-cAMP (to 6.24±2.24-fold, p>0.05 and 8.60±2.85-fold, p<0.05 at

2 and 6 hours of stimulation respectively, Figure 4.5C) stimulation. However, mRNA levels of the CREB-independent housekeeping gene 36B4 remained unaffected by any of these stimuli (Figure 4.5 D-F). Taken together, these data indicate that cAMP-elevating stimuli selectively repress SRF- and TEAD-dependent gene expression, while stimulating CREB-dependent gene expression in cardiac fibroblasts.

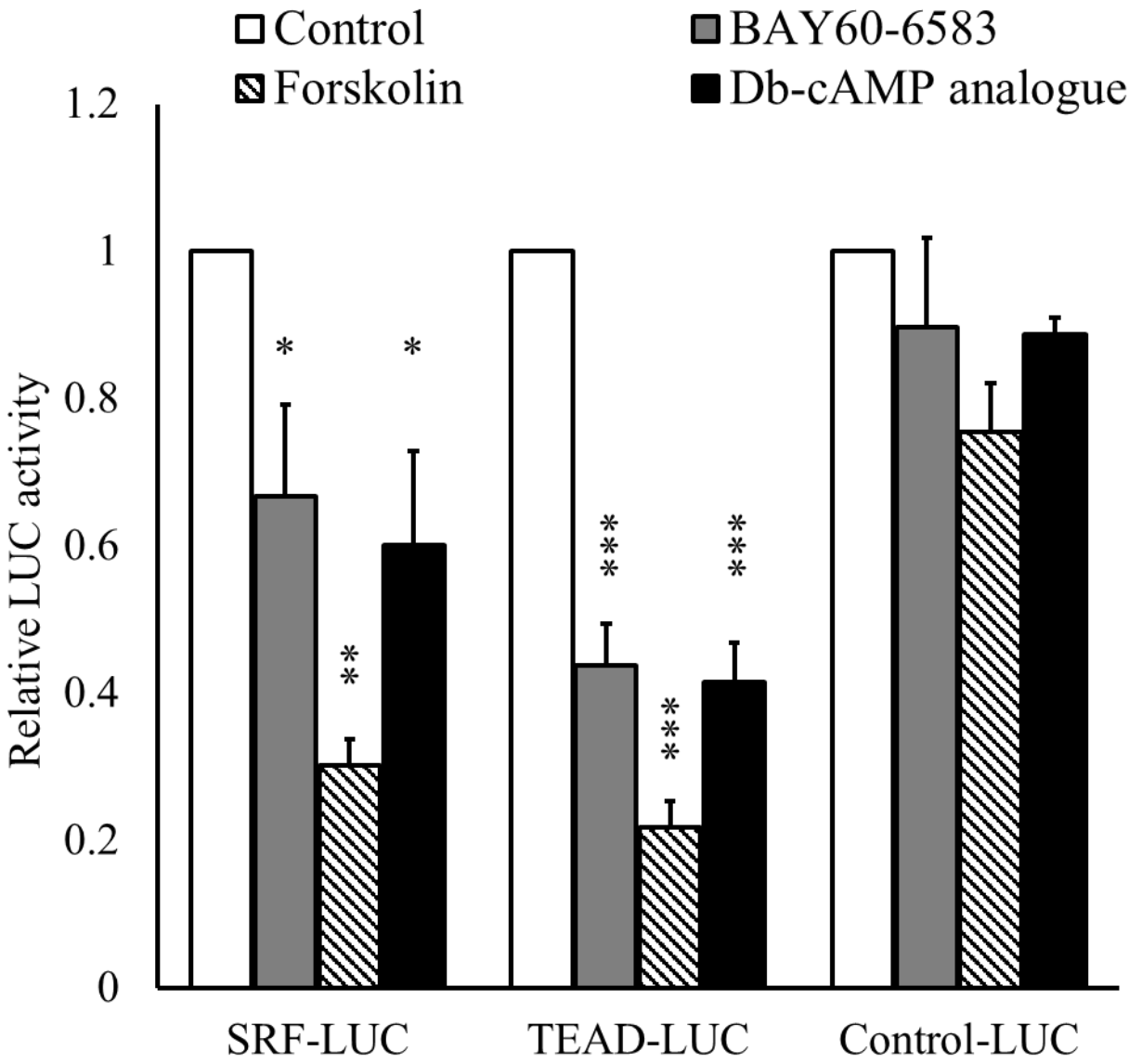


Figure 4.1: Elevated cAMP inhibits SRF and TEAD-dependent luciferase reporter gene activities in cardiac fibroblasts

Rat cardiac fibroblasts were transfected with SRF-LUC, TEAD-LUC or Control-LUC reporter plasmids and stimulated with 5 µg/ml BAY60-6583, 25 µM forskolin or 200 µM Db-cAMP analogue for 8 hours in serum free conditions and lysates assayed for luciferase activity. *: $p < 0.05$; **: 0.01 and ***: 0.001. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

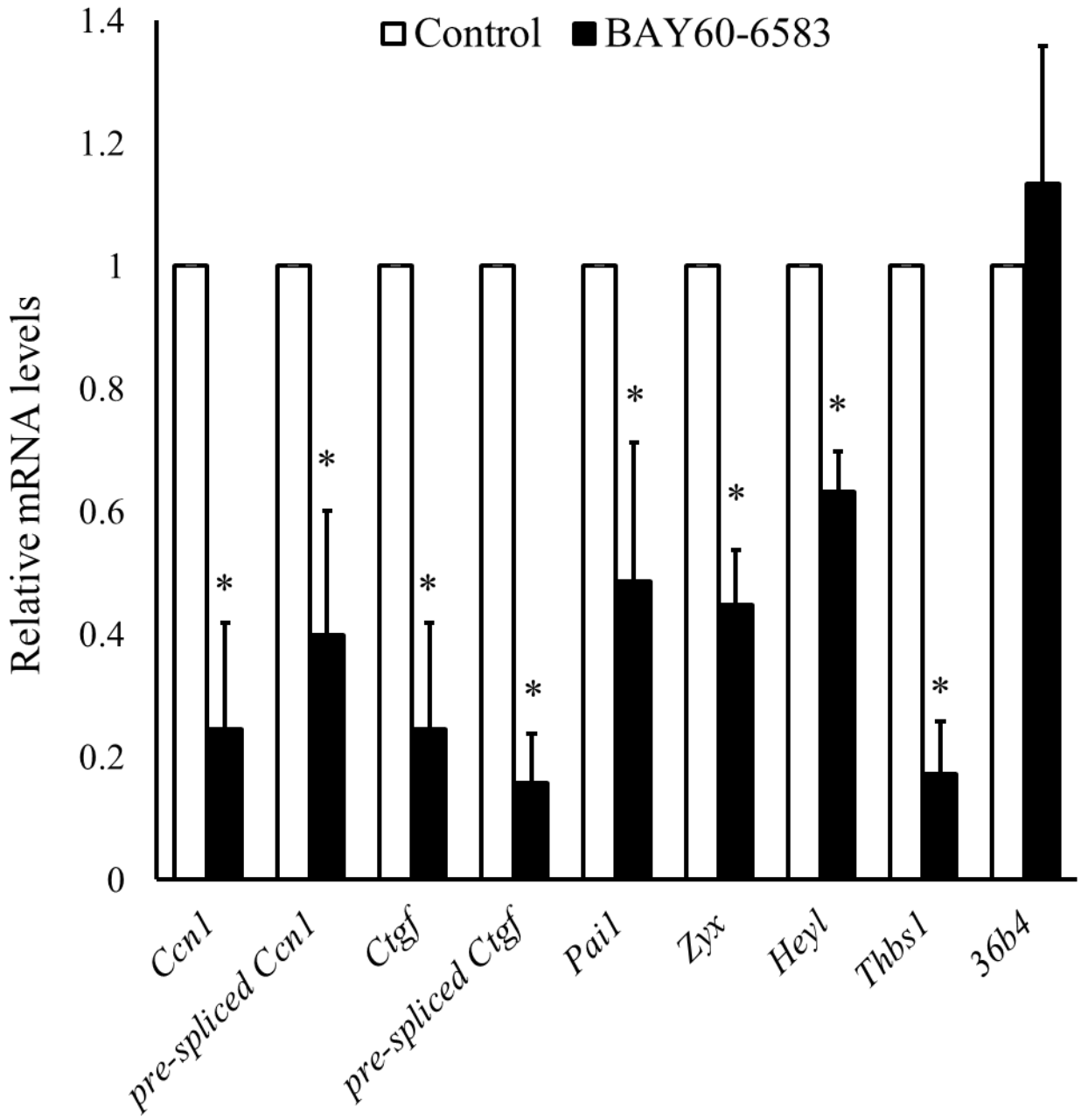


Figure 4.2: BAY60-6583 inhibits the mRNA levels of SRF and TEAD-target genes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 5 µg/ml BAY60-6583 in serum free conditions for 6 hours and mRNA levels of indicated genes quantified by RT-qPCR. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=4.

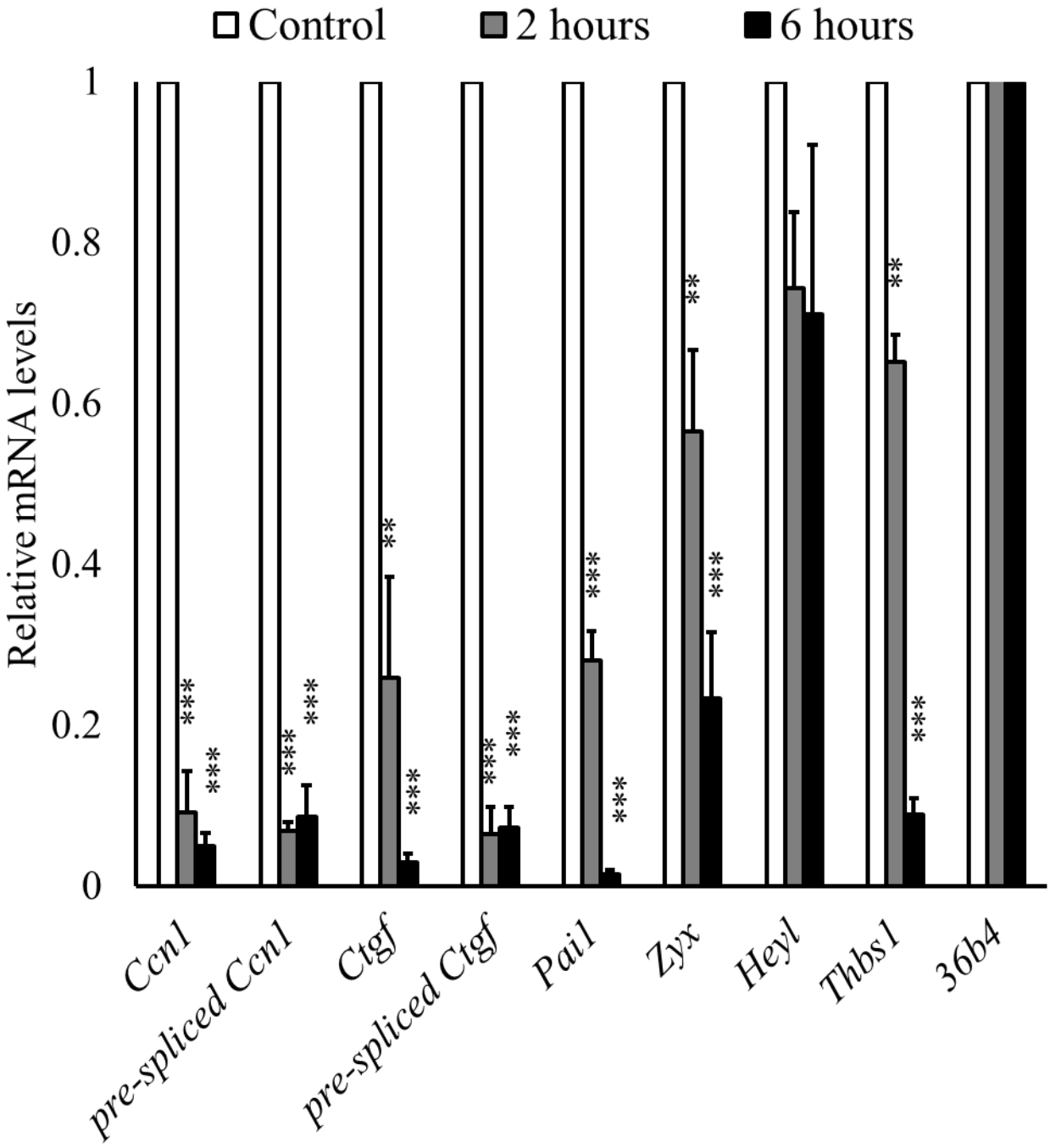


Figure 4.3: Forskolin inhibits the mRNA levels of SRF and TEAD-target genes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 25 µM forskolin in serum free conditions for 2 and 6 hours and mRNA levels of indicated genes quantified by RT-qPCR. **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

4.3.4 Elevated levels of cAMP suppress Rho/ROCK signalling in cardiac fibroblasts

As demonstrated in chapter 3, elevated levels of cAMP and pharmacological inhibition of Rho/ROCK induced morphological changes and actin remodelling in cardiac fibroblasts. Moreover, our group has also shown that, elevation of cAMP levels down regulates the activity of the Rho/ROCK signalling pathway in vascular smooth muscle cells (Smith, Hudson et al. 2017). The next question was if elevated levels of cAMP also down regulate the activity of the Rho/ROCK signalling pathway in cardiac fibroblasts and if this is involved in the inhibition of SRF- and TEAD-dependent gene expression.

In order to answer this question, cardiac fibroblasts were stimulated with BAY60-6583, forskolin or Db-cAMP analogues at different times. At the end of each time point, the protein levels of phosphorylated myosin phosphatase targeting subunit (pMYPT) were quantified by Western blotting. Briefly, RhoA mediates activation of ROCK, which then phosphorylates its downstream target, MYPT, at Thr696 and Thr853. ROCK mediated phosphorylation of MYPT results in MYPT inactivation. Upon MYPT inactivation, MLC phosphorylation levels increase, resulting in stress fibre formation and cellular contraction (Kimura, Ito et al. 1996, Birukova, Smurova et al. 2004, Birukova, Smurova et al. 2004, Amano, Nakayama et al. 2010). Protein levels of pMYPT (Thr853) were significantly down regulated in response to BAY60-6583 (to 0.39±0.14-fold, $p<0.05$ and 0.38±0.082-fold, $p<0.05$ at 40 and 60 minutes of stimulation respectively, Figure 4.6), forskolin (to 0.32±0.11-fold, $p<0.001$; 0.35±0.10-fold, $p<0.001$ and 0.26±0.11-fold, $p<0.001$ at 20, 40 and 60 minutes of stimulation respectively, Figure 4.7) or Db-cAMP analogue (to 0.20±0.01-fold, $p<0.001$; 0.33±0.14-fold, $p<0.001$ and 0.27±0.11-fold, $p<0.001$ at 20, 40 and 60 minutes of stimulation respectively, Figure 4.8). This indicates that elevated cAMP induces inhibition of RhoA/ROCK signalling in cardiac fibroblasts, which is consistent with the effects previously reported in vascular smooth muscle cells (Smith, Hudson et al. 2017).

4.3.5 Y27632 inhibits SRF and TEAD-dependent luciferase reporter gene activities in cardiac fibroblasts

Since cAMP inhibits SRF and TEAD activity in cardiac fibroblasts and this was associated with an inhibition of RhoA/ROCK signalling, we next tested if SRF and TEAD activity was dependent on ROCK activity. In order to test this, cardiac fibroblasts were transiently transfected with either SRF-LUC or TEAD-LUC reporter plasmids. These cells were then stimulated with 10 µM Y27632 for eight hours to inhibit ROCK activity (Figure 3.13), which significantly down regulated the activity of SRF-LUC (to 0.32±0.10-fold, $p<0.05$) and TEAD-LUC (to 0.44±0.09-fold, $p<0.05$) (Figure 4.9). In contrast, the activity of Control-LUC, which was under the control of a minimal promoter region

lacking SRF or TEAD binding elements, remained unaffected by Y27632 treatment (Figure 4.9). These data demonstrate that ROCK regulates the activities of SRF-LUC and TEAD-LUC in cardiac fibroblasts.

4.3.6 Y27632 down regulates the expression of SRF and TEAD-target genes in cardiac fibroblasts

Previously in this chapter (see Chapter 4, section 3.1), we showed that elevated levels of cAMP down regulated SRF-LUC and TEAD-LUC activity. Consistent with this, the mRNA levels of SRF and TEAD-target genes were also suppressed (see Chapter 4, section 3.2). Likewise, we demonstrated that elevated levels of cAMP inhibit Rho/ROCK signalling (see Chapter 4, section 3.4). We therefore asked if the inhibitory effects of cAMP signalling on the SRF and TEAD-dependent gene expression was due to cAMP-mediated ROCK inhibition. In order to answer this question, cardiac fibroblasts were treated with 10 μM Y27632. This resulted in a rapid and significant down regulation of mRNA levels of *Ccn1* (to 0.76±0.15-fold and to 0.31±0.079-fold at 2 and 6 hours respectively, $p<0.01$), *Ccn1* pre-spliced RNA (to 0.82±0.075-fold and to 0.38±0.11-fold at 2 and 6 hours respectively, $p<0.05$), *Ctgf* (to 0.53±0.16-fold and to 0.43±0.15-fold at 2 and 6 hours respectively, $P<0.05$), *Ctgf* pre-spliced RNA (to 0.58±0.14-fold and to 0.46±0.19-fold at 2 and 6 hours respectively, $p<0.001$), *Serpine/Pai1* (to 0.20±0.02-fold at 6 hours), *Zyx* (to 0.75±0.14-fold and to 0.40±0.088-fold at 2 and 6 hours respectively, $p<0.001$) and *Heyl* (to 0.33±0.046-fold at 6 hours, $p<0.05$), while the mRNA levels of *36B4* were not affected (Figure 4.10).

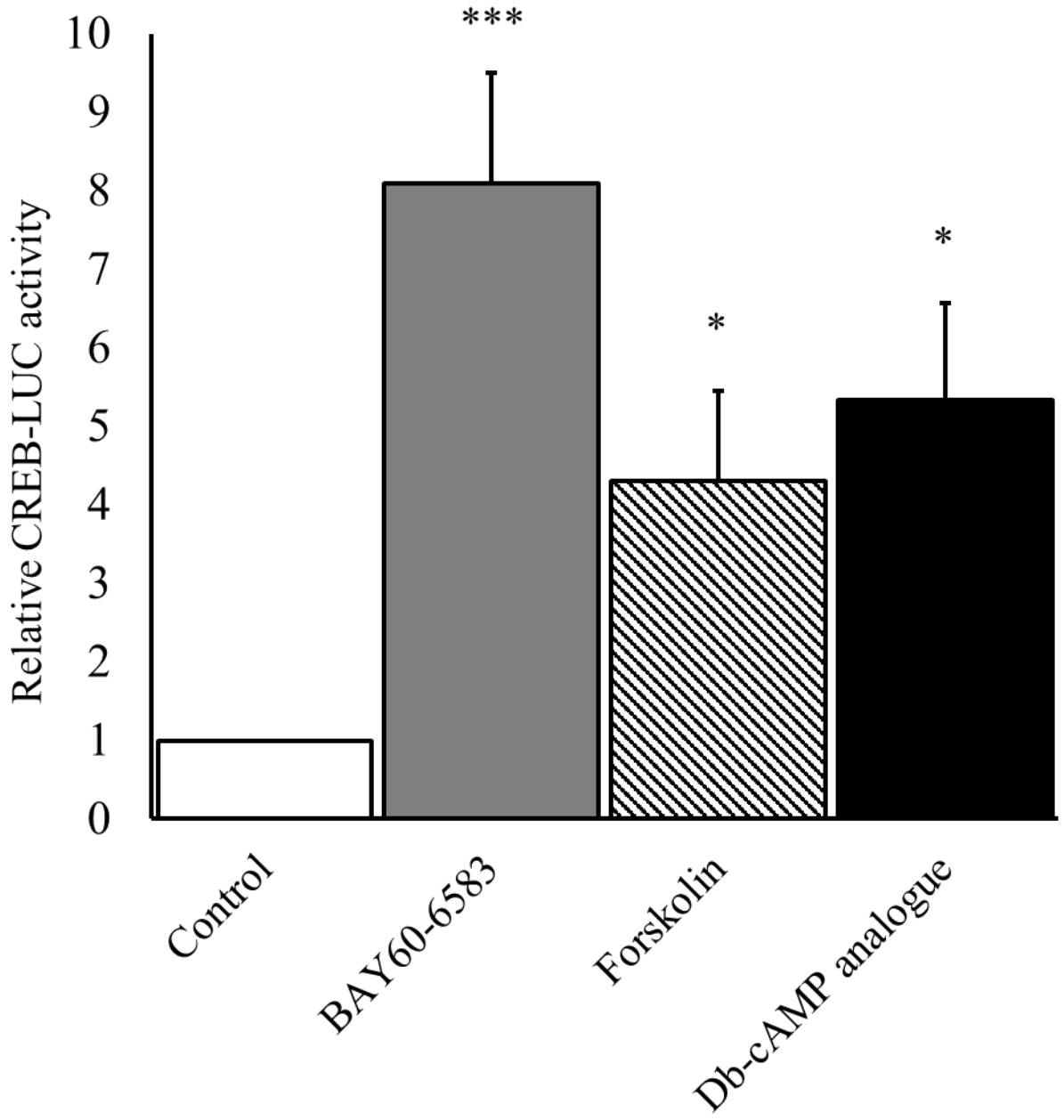


Figure 4.4: cAMP-elevating agents stimulate CREB-LUC in cardiac fibroblasts

Rat cardiac fibroblasts were transfected with CREB-LUC and stimulated with 5 µg/ml BAY60-6583, 25 µM forskolin or 200 µM Db-cAMP analogue in serum free conditions for 8 hours. The cells were lysed and their lysates assayed for the activity of luciferase. ***: $p<0.001$ and *: $p<0.05$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4

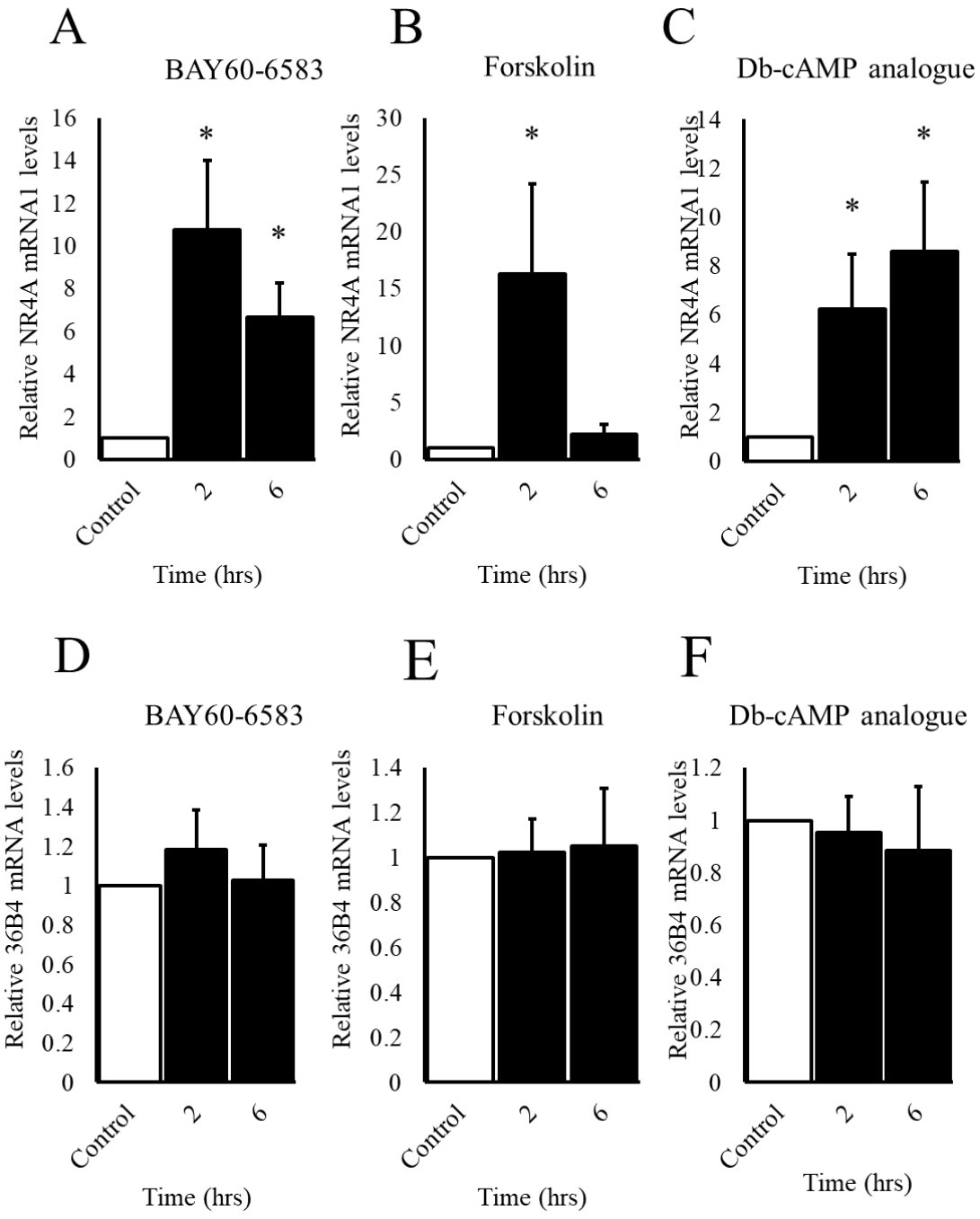


Figure 4.5: cAMP-elevating agents stimulate CREB-target gene (Nr4a1) in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 5 µg/ml BAY 60-6583 (**A** and **D**), 25 µM forskolin (**B** and **E**) and 200 µM Db-cAMP (**C** and **F**) analogue in serum free conditions for 2 and 6 hours. The mRNA levels of *Nr4a1* (**A, B** and **C**) and 36B4 (**D, E** and **F**) were quantified by RT-qPCR. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

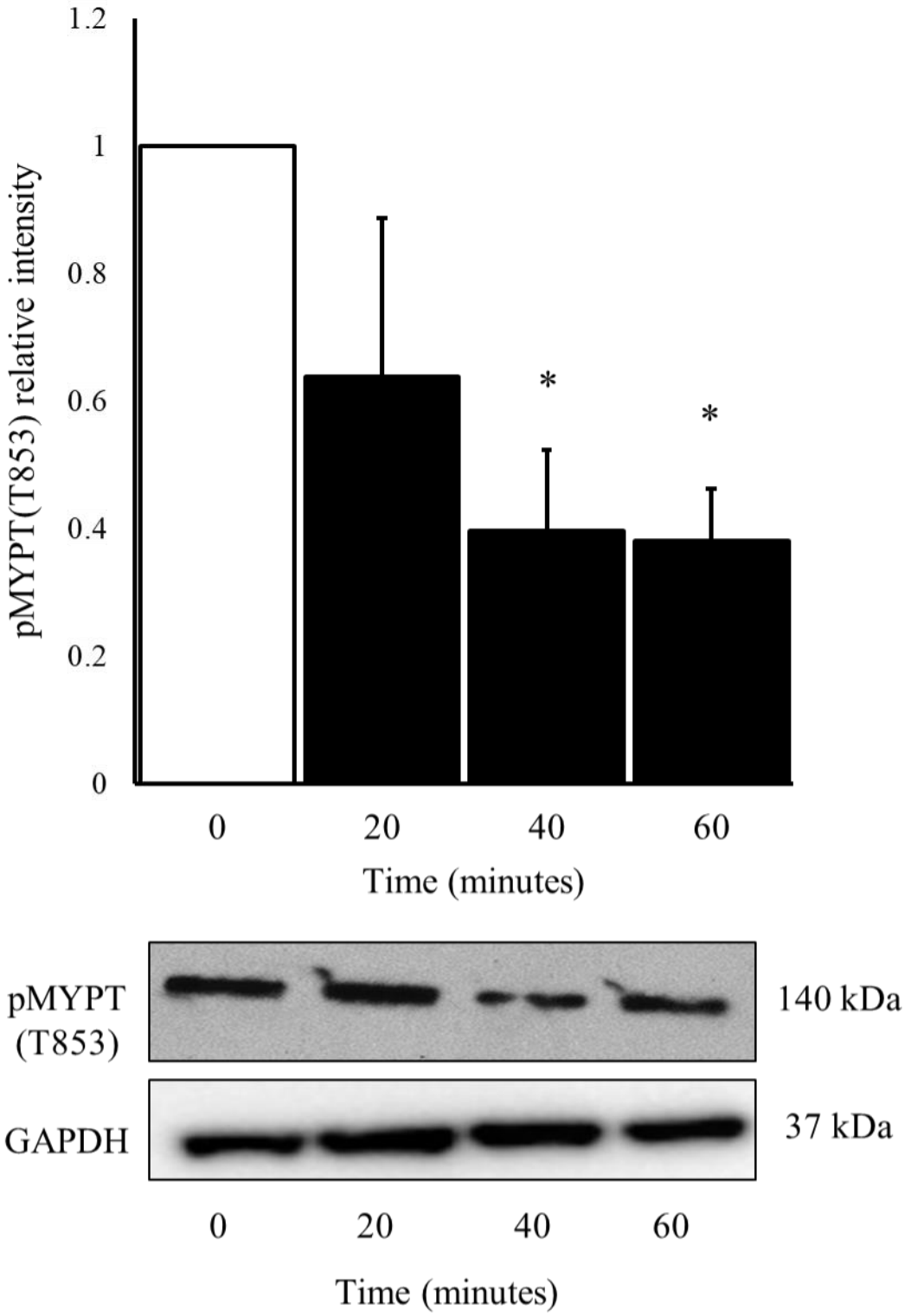


Figure 4.6: **BAY60-6583 inhibits phosphorylation of MYPT in cardiac fibroblasts.**

Rat cardiac fibroblasts were treated with 5 µg/ml BAY60-6583 for indicated times and total cell lysates analysed for phosphorylated MYPT by western blotting. Representative images of pMYPT and GAPDH Western blots are shown below the graph. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4

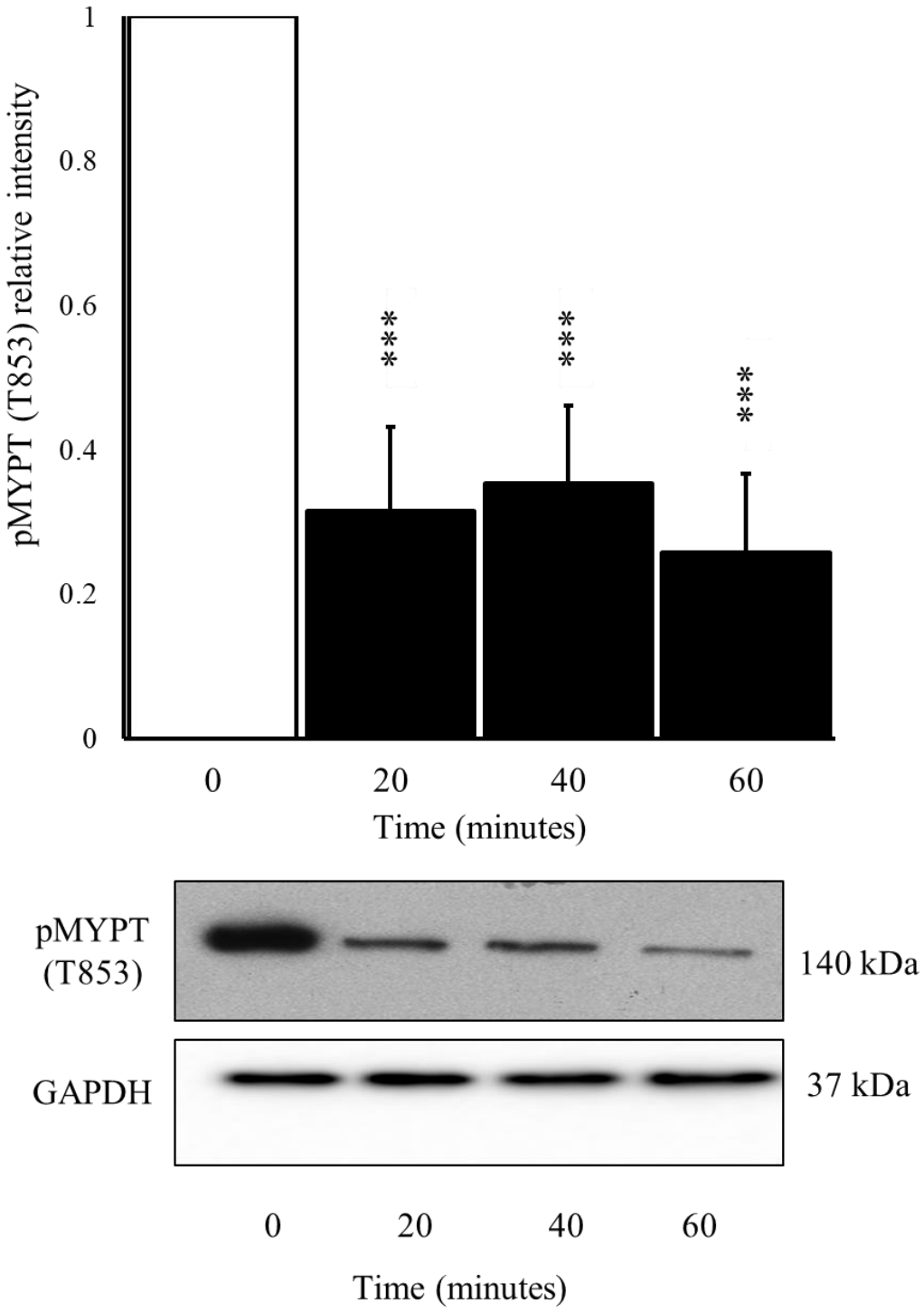


Figure 4.7: **Forskolin inhibits phosphorylation of MYPT in cardiac fibroblasts.**

Rat cardiac fibroblasts were treated with 25 µM forskolin for indicated times and total cell lysates analysed for phosphorylated MYPT and GAPDH by western blotting. Representative images of pMYPT and GAPDH Western blots are shown below the graph ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

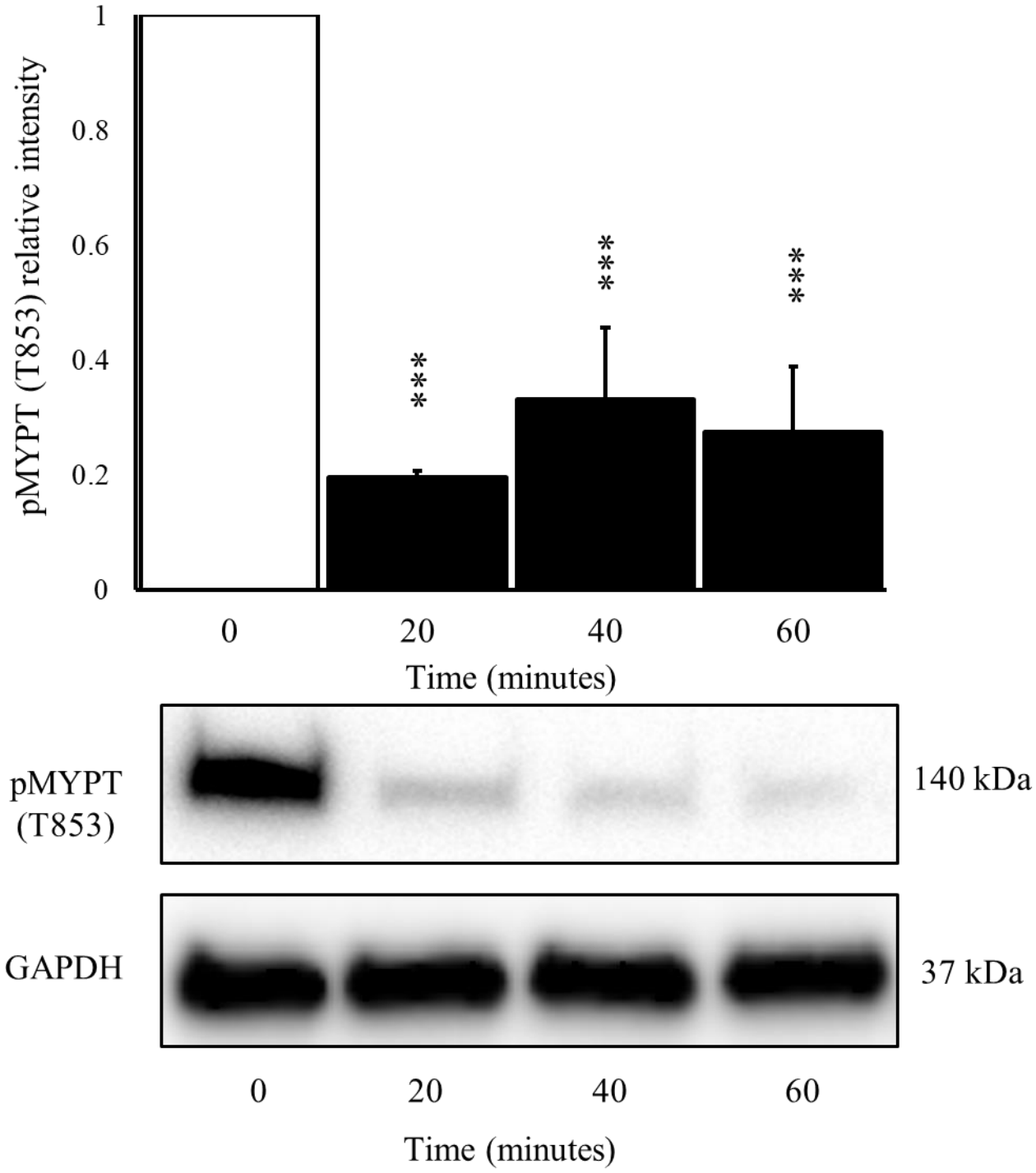


Figure 4.8: **Db-cAMP analogue inhibits phosphorylation of MYPT in cardiac fibroblasts.** Rat cardiac fibroblasts were treated with 200 µM Db-cAMP analogue for indicated times and total cell lysates analysed for phosphorylated MYPT and GAPDH by western blotting. Representative images of pMYPT and GAPDH western blots are shown below the graph. ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

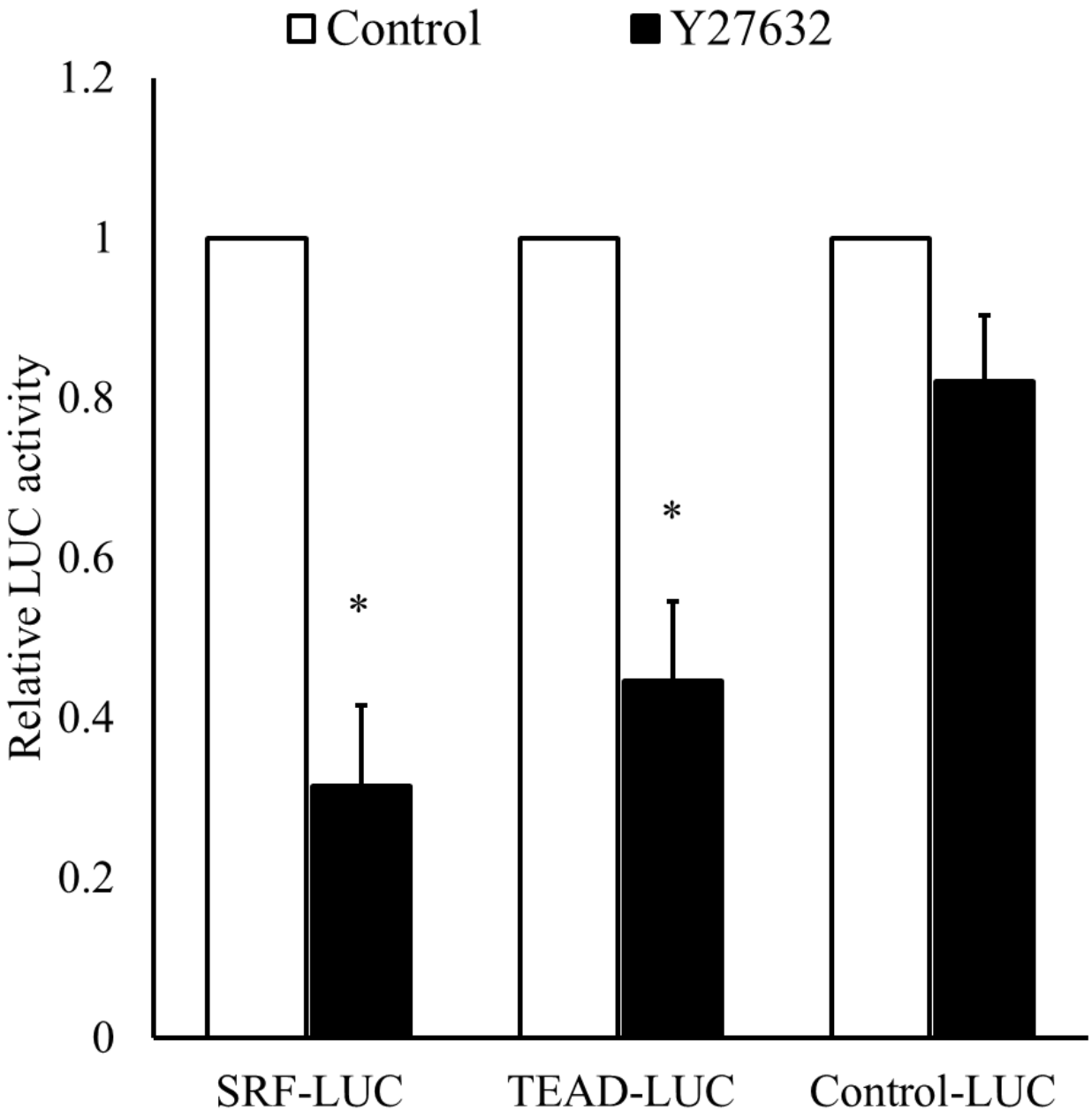


Figure 4.9: Y27632 inhibits SRF and TEAD-dependent luciferase reporter gene activities in cardiac fibroblasts

Rat cardiac fibroblasts were transfected with SRF-LUC, TEAD-LUC or Control-LUC reporter plasmids and stimulated with 10 μM Y27632 for 8 hours in serum free conditions and lysates assayed for luciferase activity. *: $p < 0.05$. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=4.

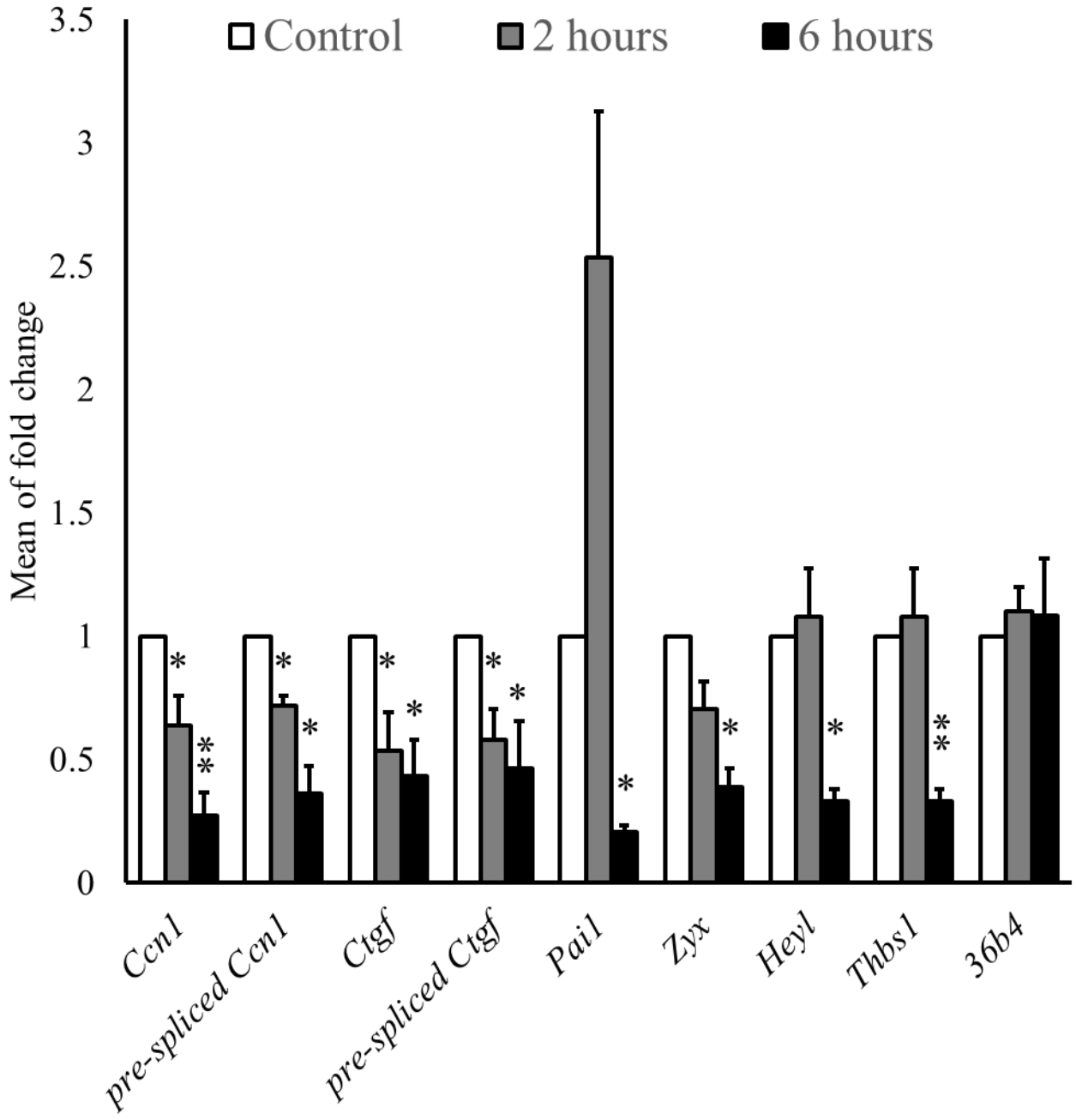


Figure 4.10: Y27632 inhibits the mRNA levels of SRF and TEAD-target genes in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 10 µM Y27632 in serum free conditions for 2 and 6 hours and mRNA levels of indicated genes quantified by RT-qPCR. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

4.3.7 cAMP elevating agents inhibits nuclear localisation of MKL1 in cardiac fibroblasts

Since the elevation of cAMP inhibited SRF-LUC reporter gene activity and down regulated the mRNA levels of SRF and TEAD-target genes in cardiac fibroblasts, we investigated the underlying mechanisms. MKL1 is one of the transcriptional co-factors for the SRF transcription factor. Nuclear localisation of MKL1 was previously reported to be sensitive to actin polymerisation, which is mediated via its N-terminal RPEL actin binding domain (Miralles, Posern et al. 2003). As shown in chapter 3, elevated cAMP induced actin depolymerisation in cardiac fibroblasts, resulting in increased levels of G-actin monomer (Figure 3.12). Moreover, our group demonstrated that depolymerisation of actin filaments and increased levels of G-actin trigger cytoplasmic localisation of MKL1 in vascular smooth muscle cells (Smith, Hudson et al. 2017). Therefore, we tested if cAMP-dependent inhibition of SRF activity in cardiac fibroblasts is mediated by inhibition of MKL1 nuclear localisation. In order to study the effects of cAMP induced actin remodelling on the cellular localisation of MKL1 in cardiac fibroblasts, cells were infected with an adenoviral vector expressing GFP-tagged MKL1 (GFP-MKL1). GFP-MKL1 expressing cardiac fibroblasts were stimulated with cAMP elevating agents for the indicated times and the sub-cellular location of MKL1 quantified. In serum starved cells, GFP-MKL1 was mostly cytoplasmic (19.4±1.16% of cells with nuclear GFP-MKL1). However, serum (FCS) stimulation triggered a rapid and significant nuclear localisation of GFP-MKL1 after only 15 minutes, which was persisted at 30 and 60 minutes after stimulation. Importantly, serum induced localisation of GFP-MKL1 was significantly inhibited by the co-stimulation of cardiac fibroblasts with BAY60-6583 at 15 (from 45.7±3.11% to 11.8±6.01% of cells with nuclear GFP-MKL1; $p<0.001$), 30 (from 48.9±2.09% to 4.78±2.85% of cells with nuclear GFP-MKL1; $p<0.001$) and 60 (from 48.0±2.48% to 6.65±4.88% of cells with nuclear GFP-MKL1; $p<0.001$) minutes (Figure 4.11 and 4.12). To test these further, cardiac fibroblasts were stimulated with forskolin and nuclear localisation of GFP-MKL1 quantified. Forskolin stimulation significantly inhibited the nuclear localisation of GFP-MKL1 after 15 minutes (from 78.6±3.14% to 32.6±8.60% of cells with nuclear GFP-MKL1; $p<0.05$), 30 minutes (from 80.5±5.45% to 11.2±2.55% of cells with nuclear GFP-MKL1; $p<0.001$) and 60 minutes (from 83.1±4.48% to 8.15±2.75% of cells with nuclear GFP-MKL1; $p<0.001$) minutes of stimulation (Figure 4.13 and 4.14).

To determine the specific roles of PKA and EPAC in the regulation of nuclear/cytoplasmic localisation of MKL1, cardiac fibroblasts were infected with an adenoviral vector expressing GFP-MKL1 and stimulated with PKA and EPAC selective cAMP analogues, alone and in combination. In the absence of serum (FCS), GFP-MKL1 was mostly cytoplasmic (38.8±5.82% of cells with

nuclear GFP-MKL1). However, PDGF stimulation triggered a rapid and increase in nuclear localisation of GFP-MKL1 after 30, 45, 60 and 75 minutes (Figures 4.14 and 4.15). Importantly, nuclear localisation of GFP-MKL1 was significantly reduced by the co-stimulation of cardiac fibroblasts with 6-BNZ-cAMP-AM at 30 (from 71.4±4.73% to 11.6±3.34% of cells with nuclear GFP-MKL1; $p<0.001$), 45 (from 81.8±6.21% to 21.9±1.89% of cells with nuclear GFP-MKL1; $p<0.001$), 60 (from 84.2±6.79% to 29.5±2.25% of cells with nuclear GFP-MKL1;$p<0.001$) and 75 (from 85.9±5.04% to 32.5±5.30%; $p<0.01$) minutes (Figures 4.14 and 4.15). Furthermore, nuclear localisation of GFP-MKL1 was also significantly reduced by the co-treatment with 8-CPT-cAMP-AM at 30 (from 71.4±4.73% to 18.0±4.91% of cells with nuclear GFP-MKL1; $p<0.001$), 45 (from 81.8±6.21% to 22.8±3.42% of cells with nuclear GFP-MKL1; $p<0.001$), 60 (from 84.2±6.79% to 43.9±10.8% of cells with nuclear GFP-MKL1;$p<0.01$) and 75 (from 85.9±5.04% to 58.9±18.1%; $p<0.05$) minutes of stimulation significantly (Figures 4.14 and 4.15). Importantly, a combination of 6-BNZ-cAMP-AM plus 8-CPT-cAMP-AM acted additively to reduce the nuclear localisation of GFP-MKL1 in cardiac fibroblasts 30 (from 71.4±4.73% to 9.85±0.43% of cells with nuclear GFP-MKL1; $p<0.001$), 45 (from 81.8±6.21% to 10.6±1.09% of cells with nuclear GFP-MKL1; $p<0.001$), 60 (from 84.2±6.79% to 13.4±3.54% of cells with nuclear GFP-MKL1;$p<0.001$) and 75 (from 85.9±5.04% to 18.6±1.96%; $p<0.01$) minutes of stimulation significantly (Figures 4.14 and 4.15). Furthermore, stimulation with the combination of 6-BNZ-cAMP-AM plus 8-CPT-cAMP-AM resulted in a significantly greater inhibition of nuclear GFP-MKL1 localisation compared to either 6-BNZ-cAMP-AM or 8-CPT-cAMP-AM alone at 60 and 75 minutes of stimulation.

4.3.8 EPAC-selective cAMP analogue 8-CPT-cAMP-AM does not stimulate PKA-sensitive gene expression

Data presented in Figure 4.16 indicates that an EPAC-selective analogues inhibit nuclear localisation of GFP-MKL1. Cytoplasmic localisation of MKL1 is typically associated with disruption of actin polymerisation and cell spreading. Interestingly, treatment of cardiac fibroblasts with EPAC-selective agonist did not induce any detectable change in cellular morphology. It is important to note that EPAC-selective cAMP analogues and their metabolites have been previously reported to have off-target effects, including inhibition of PDE activity and subsequent elevation of endogenous cAMP levels (Poppe, Rybalkin et al. 2008, Enyeart and Enyeart 2009, Sand, Grandoch et al. 2010, Herfindal, Nygaard et al. 2013, Herfindal, Krakstad et al. 2014). To test if stimulation of cardiac fibroblasts with 8-CPT-cAMP-AM may be inducing PKA activity via these potential off-target effects, we quantified the expression levels of the classical PKA inducible gene *Nr4a1*. Expression of *Nr4a1* is under the control of the CREB transcription factor and is induced in response

to PKA activation. Stimulation of cardiac fibroblasts with the PKA-selective analogues 6-BNZ-cAMP-AM resulted in a three-fold induction of *Nr4a1* mRNA levels (Figure 4.17). However, stimulation with the EPAC-selective analogues 8-CPT-cAMP-AM did not result in any detectable induction of *Nr4a1* mRNA levels. Taken together, these data indicate that 8-CPT-cAMP-AM stimulation at the dose used in these experiments does not results in an off-target activation of PKA-CREB signalling.

4.3.9 Elevated levels of cAMP inhibit nuclear localisation of YAP in cardiac fibroblasts

Since the elevation of cAMP inhibited TEAD-LUC activity and down regulated the mRNA levels of SRF and TEAD-target genes in cardiac fibroblasts, we investigated the underlying mechanisms. YAP is one of the transcriptional co-factors for TEAD transcription factor. Nuclear localisation of YAP was previously reported to be sensitive to actin polymerisation (Reddy, Deguchi et al. 2013, Das, Fischer et al. 2016, Seo and Kim 2018). As shown in chapter 3, elevated cAMP induced actin depolymerisation in cardiac fibroblasts, resulting in increased levels of G-actin monomer (Figure 3.12). Moreover, studies in vascular smooth muscle cells demonstrated that depolymerisation of actin filaments and increased levels of G-actin trigger, YAP phosphorylation, its cytoplasmic localisation and degradation (Dupont, Morsut et al. 2011). Likewise, as stimulation of cAMP signalling pathway induced similar changes in actin cytoskeleton and levels of G-actin in cardiac fibroblasts, we tested if cAMP-dependent inhibition of TEAD activity in these cells is mediated by YAP phosphorylation, its cytoplasmic localisation and degradation. In order to study this, cardiac fibroblasts were stimulated with forskolin for the indicated times. Moreover, Western blot analysis of total cardiac fibroblasts lysates demonstrated a significant and rapid reduction of total YAP protein levels (Figure 4.18A and D) and confirmed an increase in the level of YAP phosphorylation at two phosphorylation sites, serine 172 (Figure 4.18B and D) and serine 397 (Figure 4.18C and D). The upregulation in the phosphorylation of YAP protein at these two phosphorylation sites have previously been associated with YAP nuclear extrusion and degradation (Zhao, Wei et al. 2007, Zhao, Li et al. 2010). Moreover, immunofluorescent staining of forskolin treated cells detected a significant inhibition in nuclear and cytoplasmic levels of YAP protein (Figure 4.19 and 4.20). Taken together, these data demonstrate that elevated cAMP inhibit TEAD activity in cardiac fibroblasts, an effect that is mediated at least in part by YAP nuclear export and degradation.

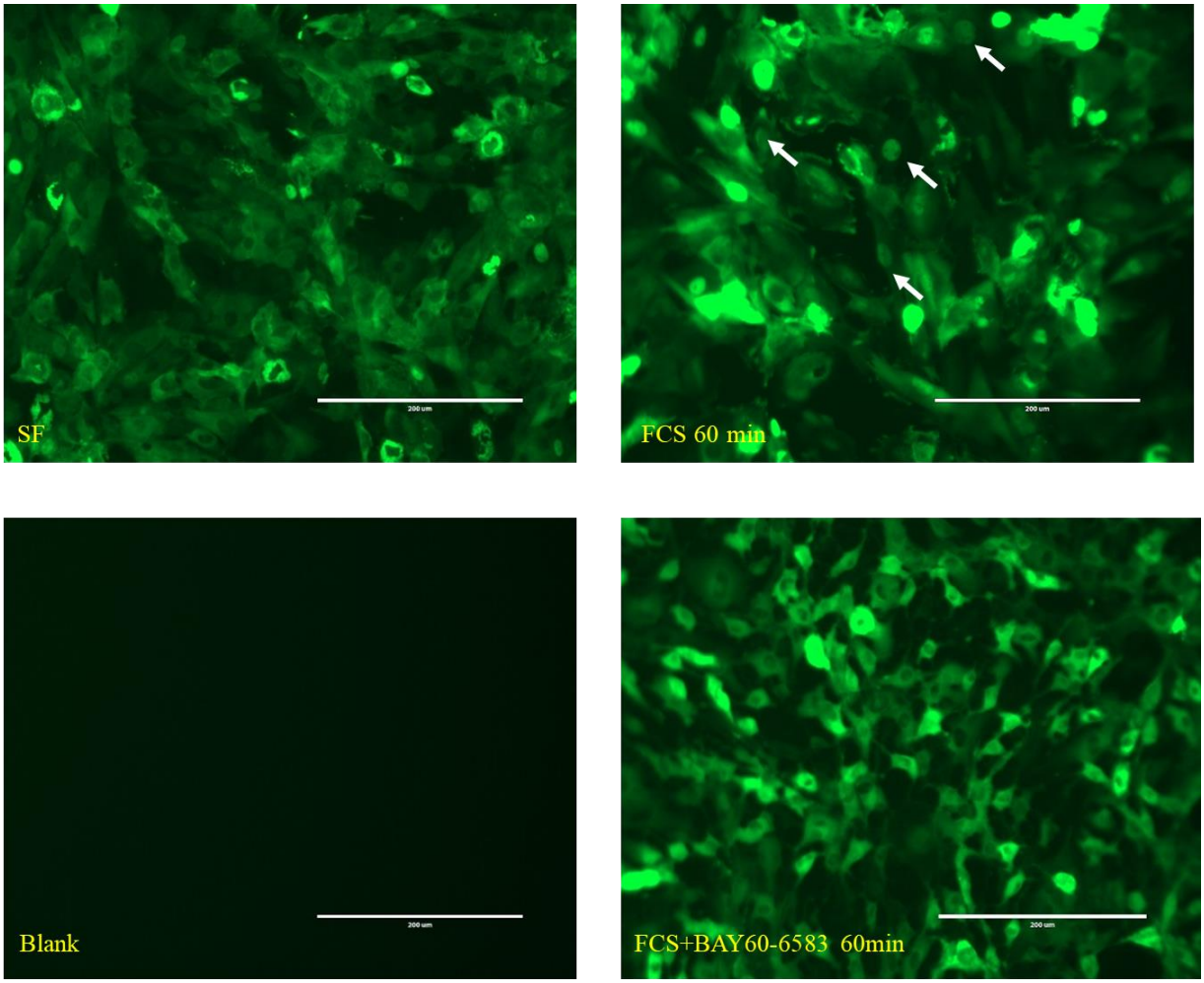


Figure 4.11: BAY60-6583 inhibits the nuclear localisation of GFP-MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were infected with an adenoviral vector expressing GFP-MKL1. Cells were serum starved overnight before being stimulated for 1 hour with 10% (v/v) FCS in the presence of 5 µg/ml BAY60-6583 (representative images, only one time point is shown). SF: serum free; FCS: Foetal calf serum; Blank: No virus; White arrows: Nuclear GFP-MKL1. Bar indicates 100 µm.

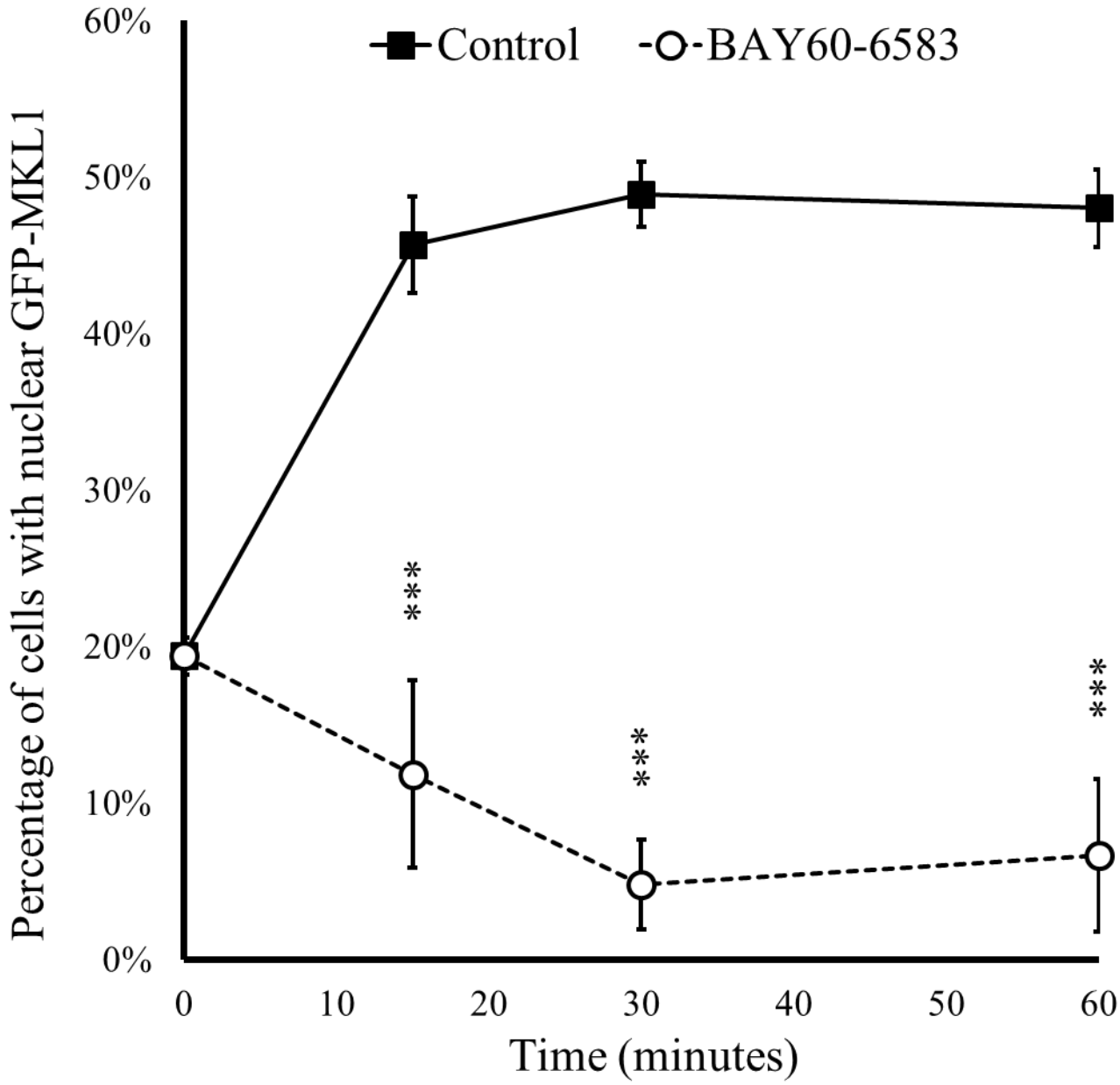


Figure 4.12: BAY60-6583 extrudes nuclear GFP-MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were infected with adenoviral vectors expressing GFP-MKL1. Cells were serum starved overnight before being stimulated for 1 hour with 10% (v/v) FCS in the presence of 5 µg/ml BAY60-6538. Cells were analysed for cellular localisation of GFP-MKL1 by fluorescence microscopy. Cellular localisation of GFP-MKL1 was quantified by image analysis ***: $p < 0.001$ when comparing BAY60-6538 to control at each time point. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

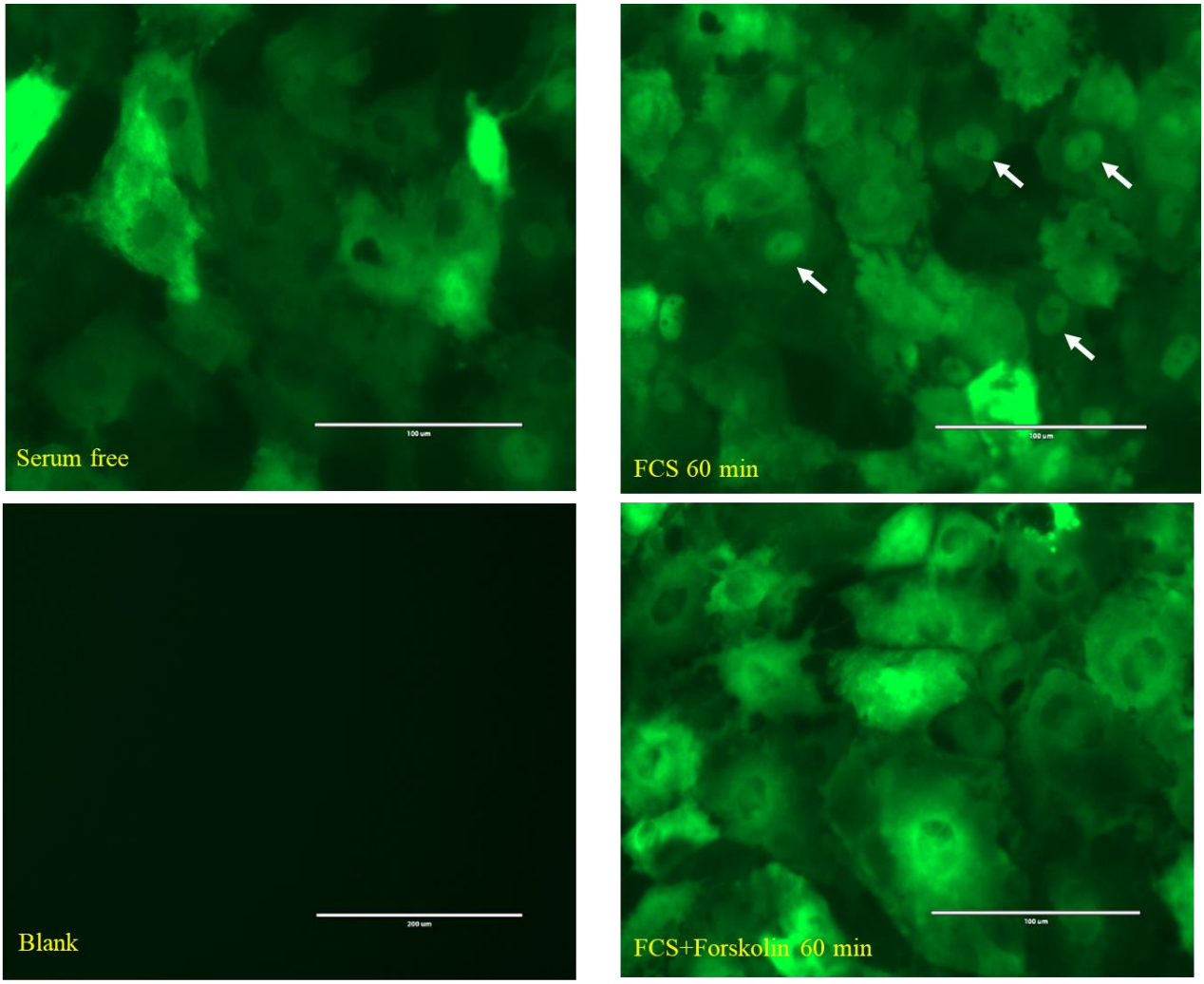


Figure 4.13: Forskolin inhibits the nuclear localisation of GFP-MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were infected with an adenoviral vector expressing GFP-MKL1. Cells were serum starved overnight before being stimulated for 1 hour with 10% (v/v) FCS in the presence of 25 µM forskolin (representative images, only one time point is shown). FCS: Foetal calf serum; Blank: No virus; White arrows: Nuclear GFP-MKL1 and min: minutes. Bar indicates 100 µm.

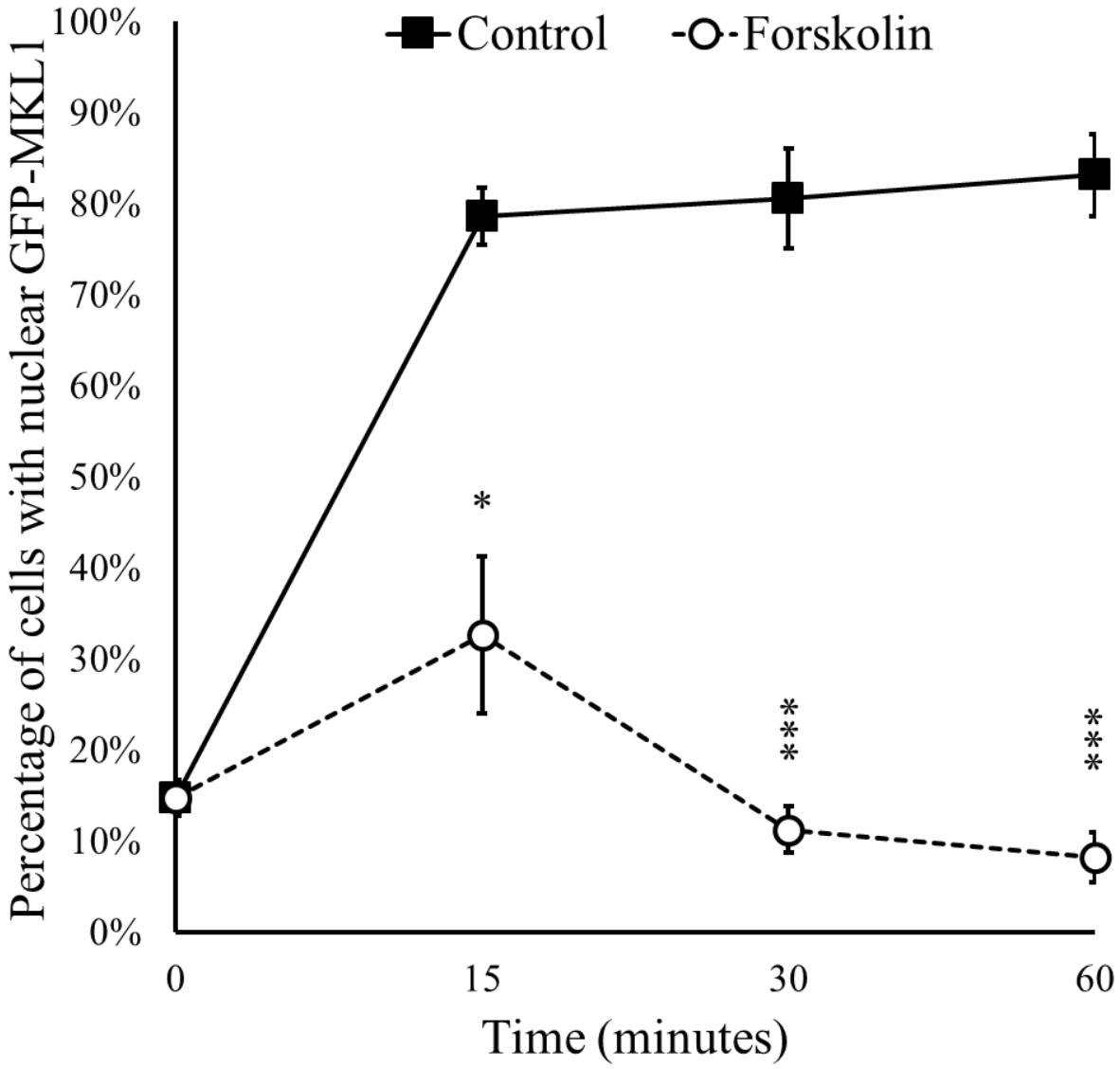


Figure 4.14: Forskolin extrudes nuclear GFP-MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were infected with an adenoviral vector expressing GFP-MKL1 (Ad:GFP-MKL1). Cells were serum starved overnight before being stimulated for 1 hour with 10% (v/v) FCS in the presence of 25 µM forskolin. Cells were analysed for cellular localisation of GFP-MKL1 by fluorescence microscopy. Cellular localisation of GFP-MKL1 was quantified by image analysis ***: $p < 0.001$ with respect to nuclear localisation. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

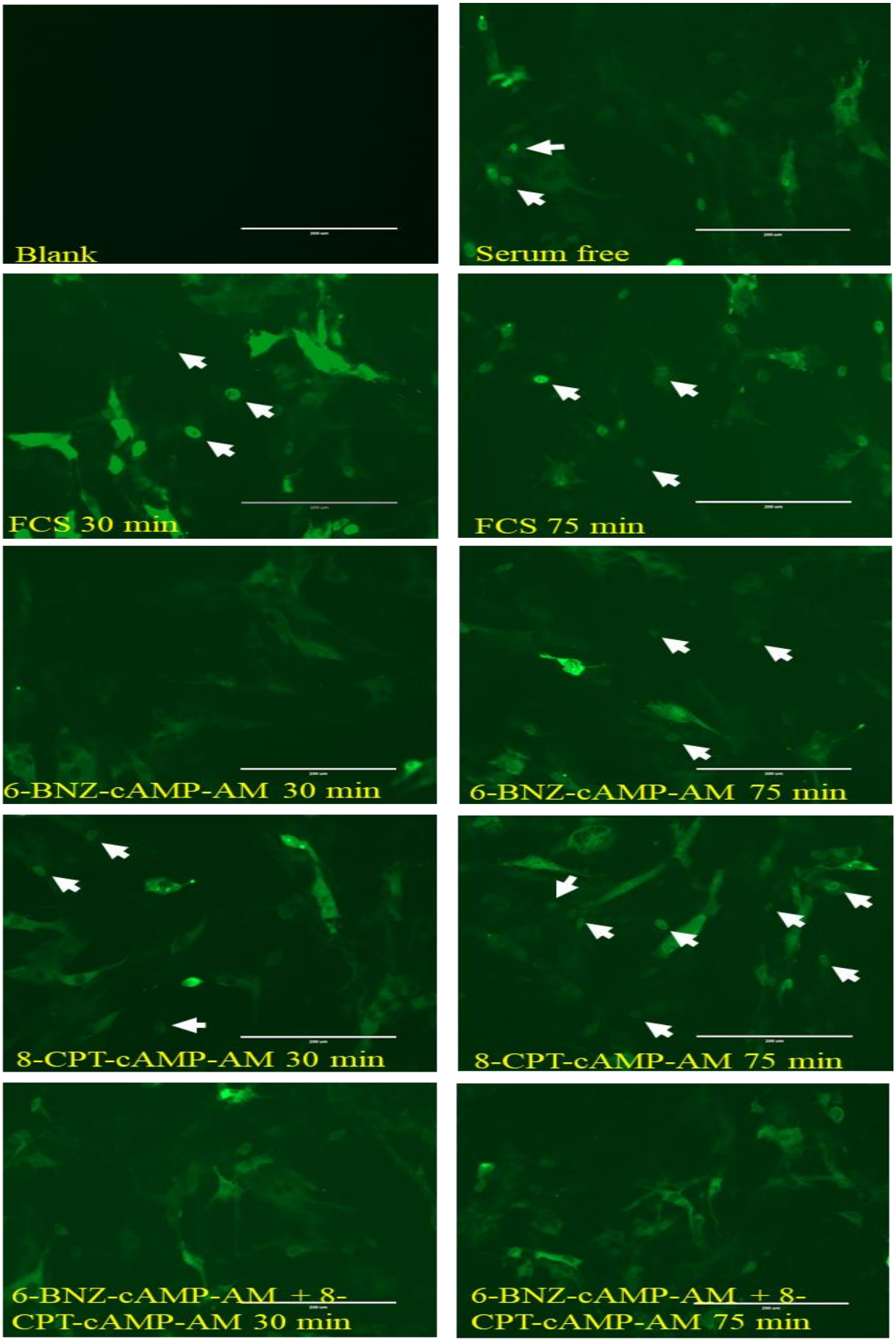

Blank
Serum free
FCS 30 min
FCS 75 min
6-BNZ-cAMP-AM 30 min
6-BNZ-cAMP-AM 75 min
8-CPT-cAMP-AM 30 min
8-CPT-cAMP-AM 75 min
6-BNZ-cAMP-AM + 8-CPT-cAMP-AM 30 min
6-BNZ-cAMP-AM + 8-CPT-cAMP-AM 75 min

Figure 4.15: 6-BNZ-cAMP-AM and 8-CPT-cAMP-AM inhibit the nuclear localisation of GFP-MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were infected with an adenoviral vector expressing GFP-MKL1 (Ad:GFP-MKL1). Cells were serum starved overnight before being stimulated for 15, 30, 45, 60 and 75 minutes with 10% (v/v) FCS in the presence of 20 µM of 6-BNZ-cAMP-AM alone, 20 µM of 8-CPT-cAMP-AM alone and 6-BNZ-cAMP-AM and 8—CPT-cAMP-AM together (representative images, only one time point is shown). FCS: Foetal calf serum; Blank: No virus; White arrows: Nuclear GFP-MKL1 and min: minutes. Bar indicates 200 µm. Due to the shortage of space, only some of the time points are shown.

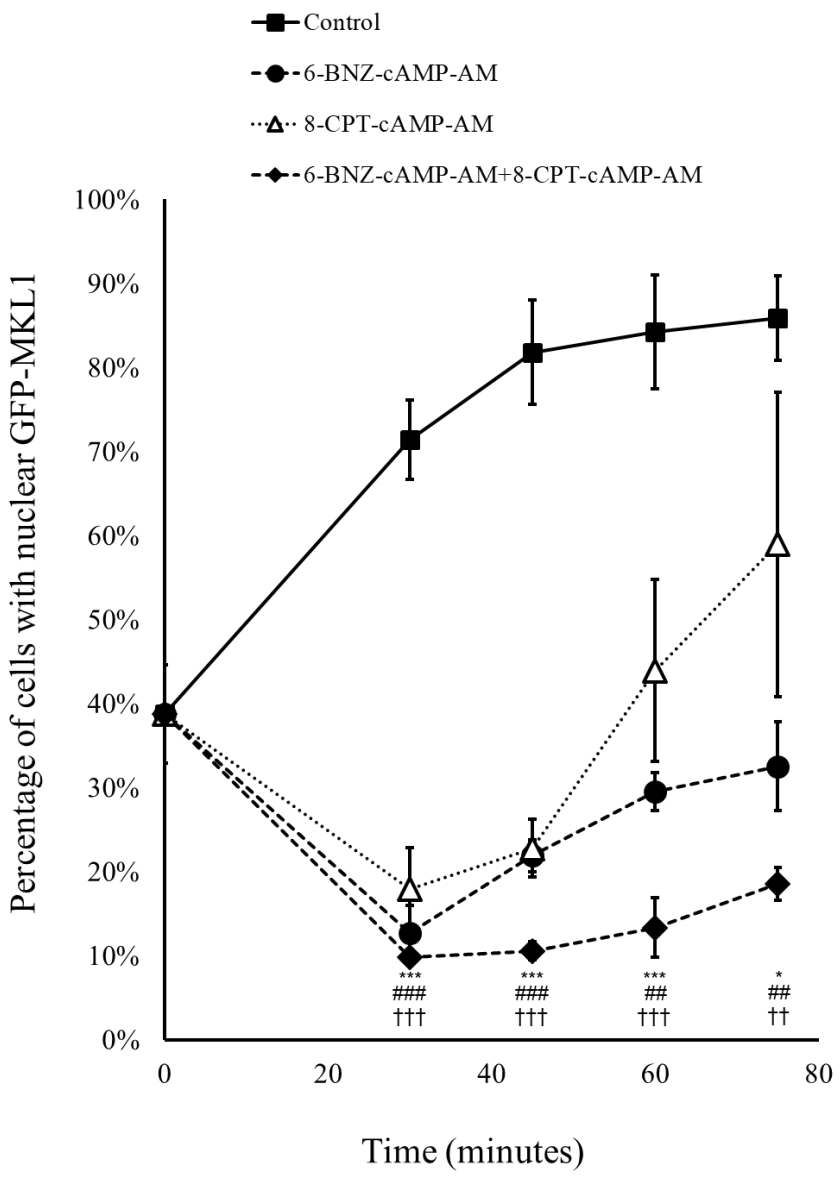


Figure 4.16: 6-BNZ-cAMP-AM and 8-CPT-cAMP-AM extrudes nuclear GFP-MKL1 in cardiac fibroblasts

Cardiac fibroblasts were infected with adenoviral vectors expressing GFP-MKL1 (Ad:GFP-MKL1). Cardiac fibroblasts were serum starved overnight before being stimulated for 1 hour with 50 ng/ml PDGF in the presence of 20 µM 6-BNZ-cAMP-AM and 20 µM 8-CPT-cAMP-AM. Cells were analysed for cellular localisation of GFP-MKL1 by fluorescence microscopy and quantified by image analysis. *: $p<0.05$ control vs 6-BNZ-cAMP-AM stimulation; **: $p<0.01$ control vs 6-BNZ-cAMP-AM stimulation;***: $p<0.001$ control vs 6-BNZ-cAMP-AM stimulation; ##: $p<0.01$ control vs 8-CPT-cAMP-AM; ###: $p<0.001$ control vs 8-CPT-cAMP-AM; ††: $p<0.01$ control vs 6-BNZ-cAMP-AM + 8-CPT-cAMP-AM; †††: $p<0.001$ control vs 6-BNZ-cAMP-AM + 8-CPT-cAMP-AM. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

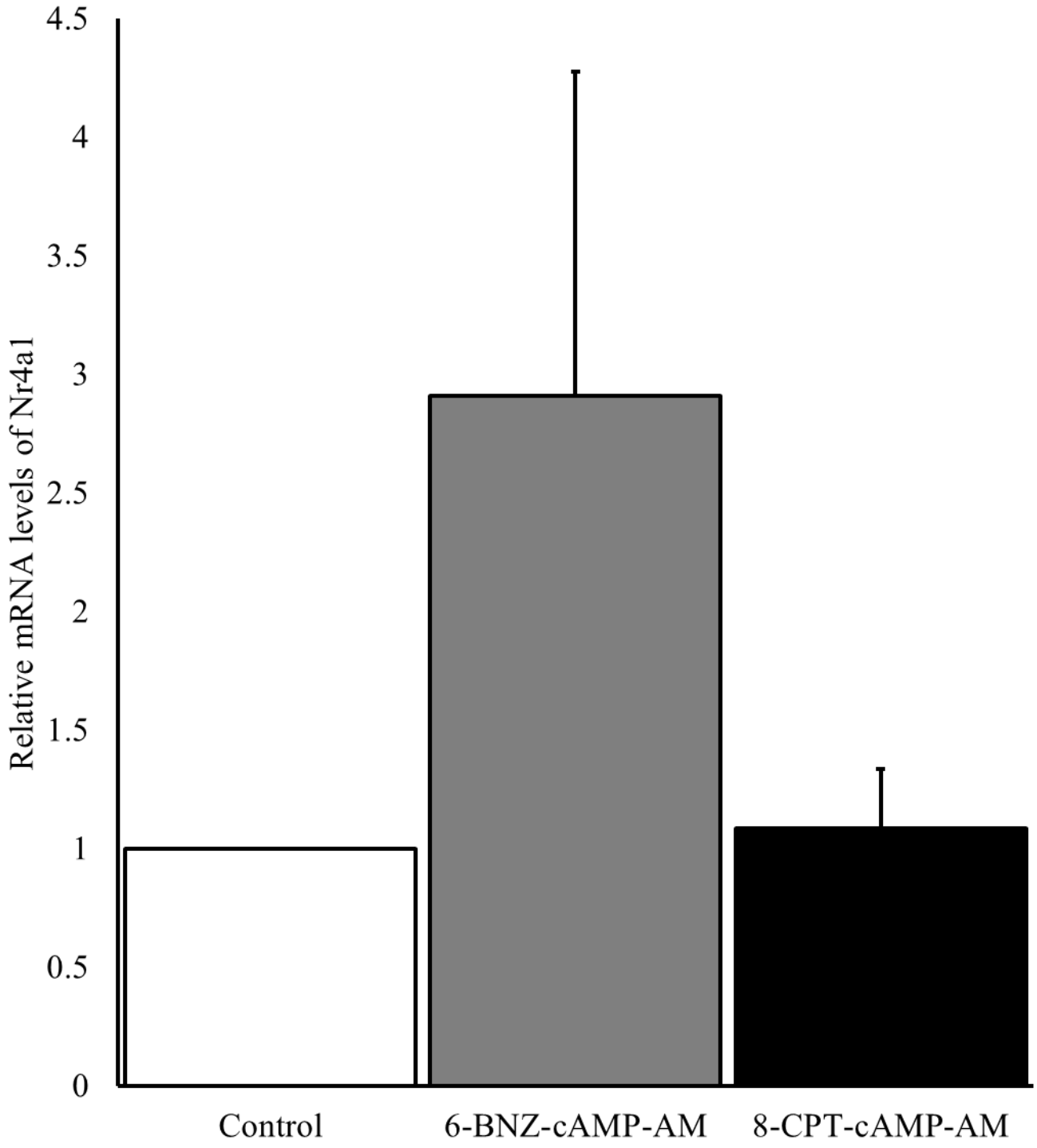


Figure 4.17: 8-CPT-cAMP-AM does not activate PKA-sensitive gene expression

Rat cardiac fibroblasts were stimulated with 5 µg/ml BAY 60-6583, 25 µM forskolin or 200 µM Db-cAMP analogue in serum free conditions for 6 hours. The mRNA levels of *Nr4a1* were quantified by RT-qPCR. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test. n=4.

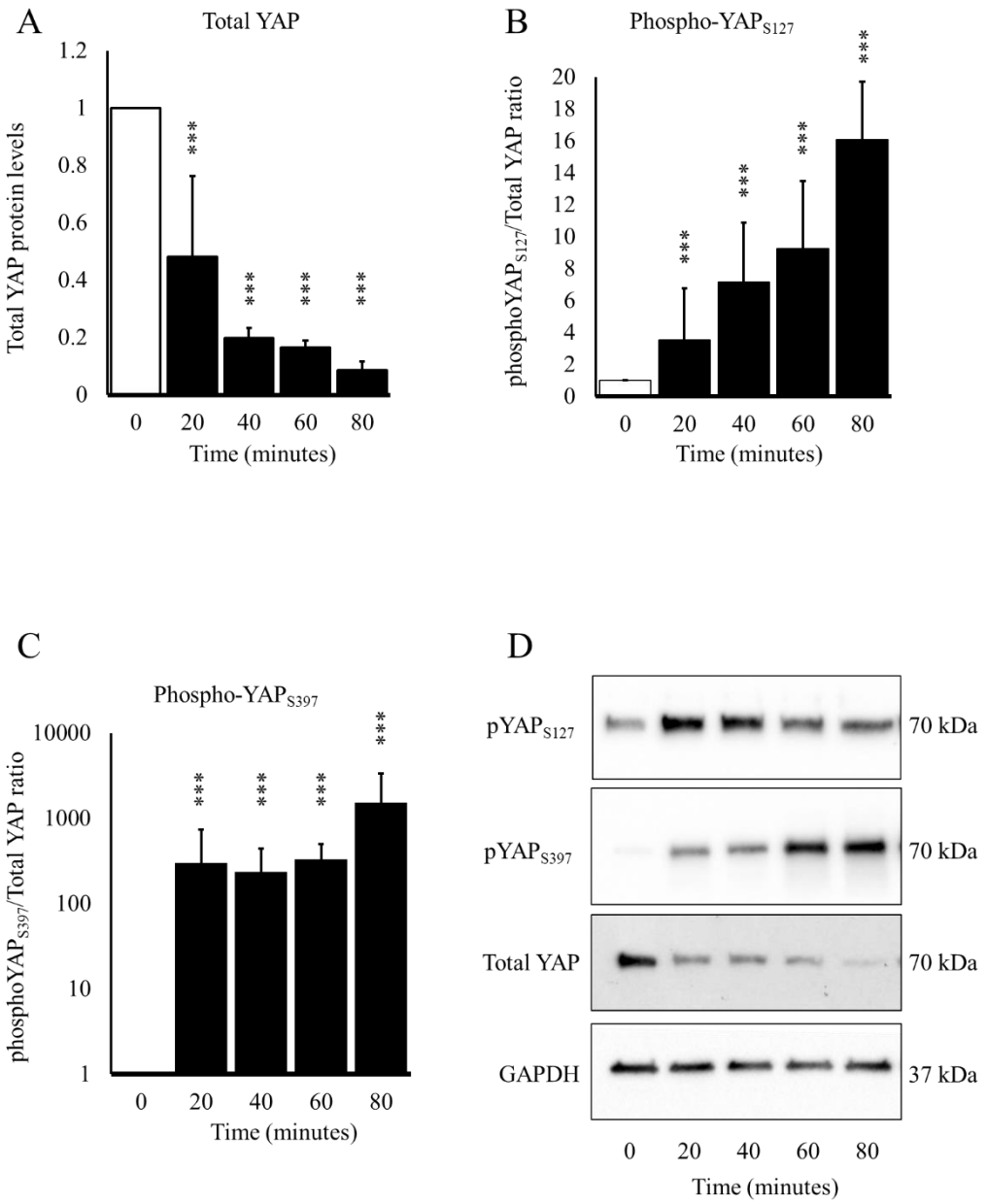


Figure 4.18: Elevated levels of cAMP inhibit total YAP protein levels in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 25 µM forskolin for the indicated times (**A**-**D**). Total cellular YAP (**A**), phospho-YAP$_{S127}$ (**B**), phospho-YAP$_{S397}$ (**C**) were quantified by Western blotting (**D**) and densitometric analysis (**A**-**C**). ***: $p<0.01$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=4.

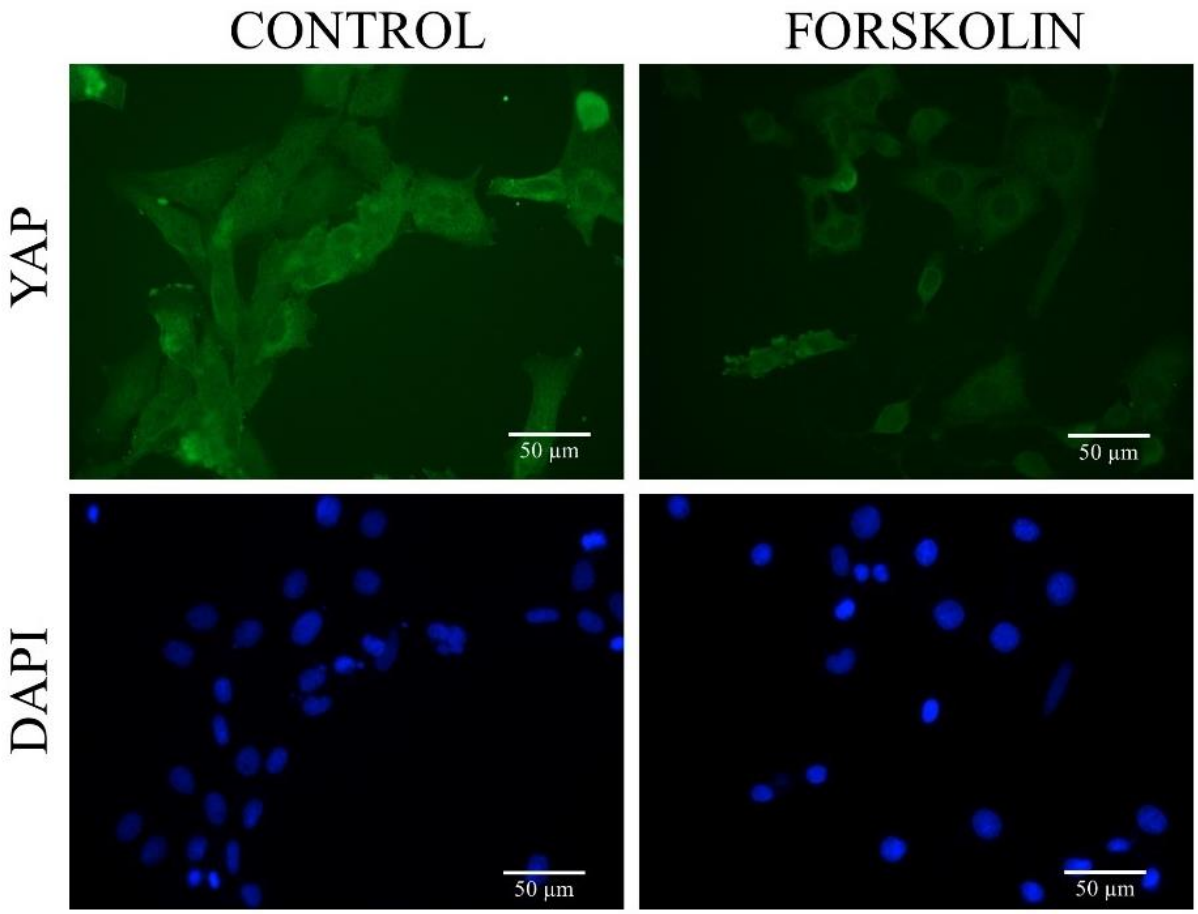


Figure 4.19: Elevated levels of cAMP inhibit nuclear localisation of YAP in cardiac fibroblasts

Rast cardiac fibroblasts were stimulated with 25 µM forskolin for 2 hours. Cells were fixed and analysed by immunofluorescent staining for YAP (green). Cells were counter stained with DAPI (blue) to visualise the nuclei.

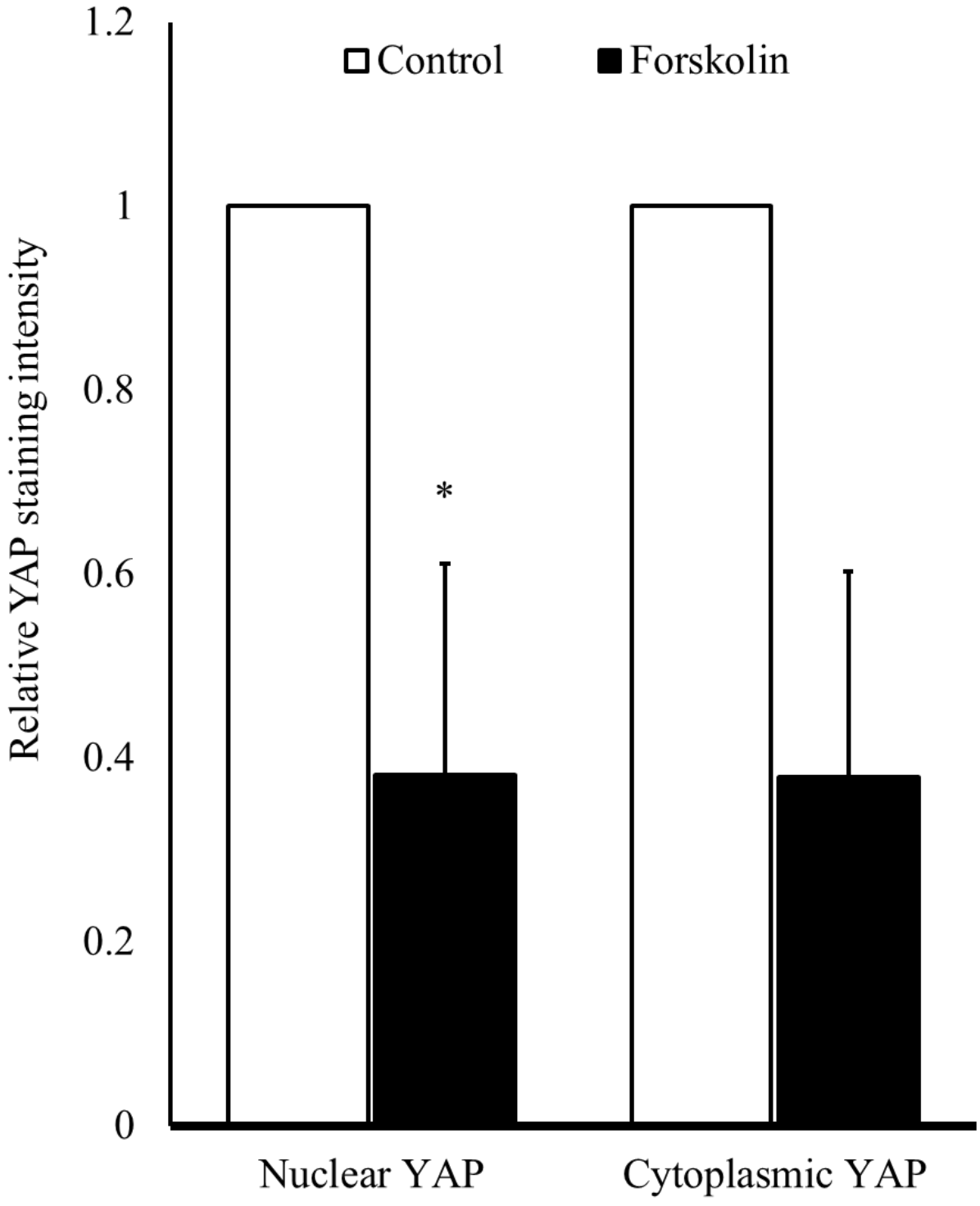


Figure 4.20: Elevated levels of cAMP inhibit total YAP protein levels in cardiac fibroblasts

Rat cardiac fibroblasts were stimulated with 25 µM forskolin for 2 hours. Nuclear and cytoplasmic YAP levels were quantified by image analysis of immunofluorescent micrographs. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

4.3.10 CCG203971 down regulates the proliferation of cardiac fibroblasts

Taken together, the data presented demonstrates that elevated levels of cAMP induce depolymerisation of the actin cytoskeleton, inhibit the nuclear localisation of MKL1, inhibits nuclear localisation of YAP, down regulate SRF/TEAD activities and SRF /TEAD-dependent gene expression. We therefore asked if inhibition of MKL1 nuclear localisation and SRF activity was involved in mediating the anti-mitogenic effects of cAMP in cardiac fibroblasts. Initially we used the second generation MKL1/2 inhibitor, known as CCG203971, with lower cytotoxicity compared to the first generation (Johnson, Rodansky et al. 2014), to test the role of MKL-SRF signalling (Evelyn, Bell et al. 2010, Bell, Haak et al. 2013). CCG203971 binds to the RPEL domains of MKL1/2, preventing their nuclear translocation, thus reducing SRF-dependent gene expression (Evelyn, Wade et al. 2007, Evelyn, Bell et al. 2010, Bell, Haak et al. 2013, Smith, Hudson et al. 2017, Yu-Wai-Man, Spencer-Dene et al. 2017). Asynchronously proliferating cardiac fibroblasts were treated with 20 μM CCG203971 for 24 hours. Cells were labelled with EdU for the last 6 hours of this incubation to label cells in S-phase of the cell cycle. Stimulation of cardiac fibroblasts with CCG203971 significantly down regulated incorporation of Edu (to 14.0±2.03%, $p<0.05$; Figure 4.21), indicating that MKL-SRF activity is essential for maximal cardiac fibroblast proliferation.

4.3.11 Silencing of MKL1 and MKL2 down regulate proliferation of cardiac fibroblasts

Inhibition of Edu incorporation with CCG203971 implies a role for MKL1/2 in the proliferation of cardiac fibroblasts. However, it should be noted that CCG203971 may also bind to the RPEL domains of other proteins RPEL-domain containing proteins (Bell, Haak et al. 2013, Hayashi, Watanabe et al. 2014, Johnson, Rodansky et al. 2014). Therefore, to further validate the inhibitory effects of CCG203971 on the proliferation of cardiac fibroblasts, MKL1 and MKL2 were silenced using small interfering RNAs (siRNA). Transient transfection of cardiac fibroblasts with siRNAs targeting MKL1 and MKL2 (siMKL1/2) significantly reduced the protein levels of MKL1 and MKL2 detected by Western blotting, compared to cardiac fibroblasts transfected with control siRNA (siNEG), without affecting the protein levels of the housekeeping gene GAPDH (Figures 4.22 and 4.23). In the context of the reporter gene assay, silencing of MKL1 and MKL2 significantly reduced the SRF-reporter gene activity (to 0.44±0.86%, $p<0.05$), without affecting the control promoter activity (Figure 4.24), consistent with the large reduction in MKL1 and MKL2 protein levels. To test the function of MKL1 and MKL2 in cardiac fibroblast proliferation, cells were transfected with siRNAs targeting MKL1 and MKL2 and labelled with EdU 48 hours after transfection. Simultaneous silencing of both MKL1 and MKL2 significantly reduced incorporation

of Edu (to 19.7±0.87%, $p<0.01$ EdU incorporation; Figure 4.25), indicating that MKL1/2 are required for maximal proliferation of cardiac fibroblasts.

4.3.12 Pharmacological TEAD inhibition with compound 3.1 reduced cardiac fibroblast proliferation

As mentioned previously in chapter 1 and demonstrated previously by our group (Kimura, Duggirala et al. 2016), elevated levels of cAMP inhibits the nuclear localisation of YAP and TAZ, two co-transcription factors of TEAD, and down regulate TEAD activity (Kimura, Duggirala et al. 2016). Likewise, we previously showed that pharmacological inhibition of YAP-TEAD activity using a novel YAP-TEAD protein:protein interaction inhibitor, termed compound 3.1 (CPD3.1), down regulates the proliferation and migration of vascular smooth muscle and HeLa cells (Smith, Sessions et al. 2019). In addition, several line of evidence demonstrated that YAP/TAZ-TEAD regulates the transcription of genes (such as, *CCN1* and *CTGF*) involved in proliferation and cell growth or various cell types (Zhang, Smolen et al. 2008, Zhao, Ye et al. 2008, Holden and Cunningham 2018, Smith, Sessions et al. 2019). Therefore, we hypothesised that the anti-mitogenic effects of cAMP in cardiac fibroblasts may, at least in part, be mediated by inhibition of YAP-TEAD activity. To test this, we pharmacologically inhibited YAP/TAZ-TEAD activity using compound 3.1 (abbreviated to CPD3.1) and quantified effects on cardiac fibroblast proliferation. To this end, asynchronously proliferating cardiac fibroblasts were treated with 60 µM CPD3.1 for 24 hours, in the presence of EdU for the last 6 hours to label cells progressing through S-phase of the cell cycle. Although we demonstrated that CPD3.1 inhibits the activity of TEAD reporter in vascular smooth muscle cells (Smith, Sessions et al. 2019), we also further confirmed this in cardiac fibroblasts (Figure 5.23). Incubation of cardiac fibroblasts with CPD3.1 significantly down regulated incorporation of Edu (to 7.16±1.46%, $p<0.05$; Figure 4.26), indicating that YAP-TEAD activity is required for maximal cardiac fibroblast proliferation.

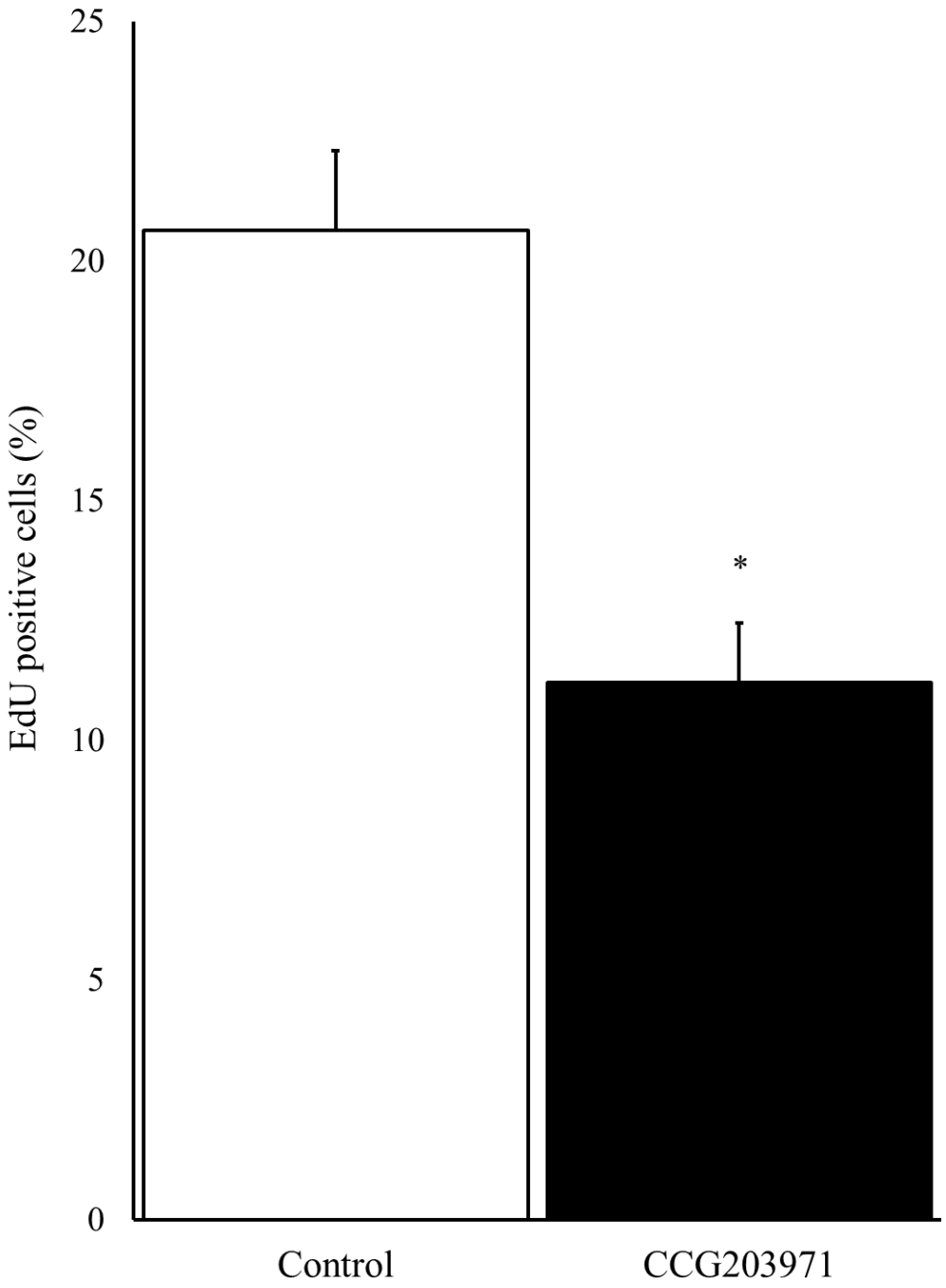


Figure 4.21: CCG203971 inhibits serum-stimulated proliferation of rat cardiac fibroblasts

Rat cardiac fibroblasts were stimulated for 24 hours with 20 µM CCG203971 in 5% (v/v) serum, as indicated. Cells were labelled with 10 µM EdU for further 6 hours and proliferation quantified by immuno-histochemical staining of incorporated Edu. *: $p<0.05$ and CCG203971: MKL1/2 pharmacological inhibitor. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

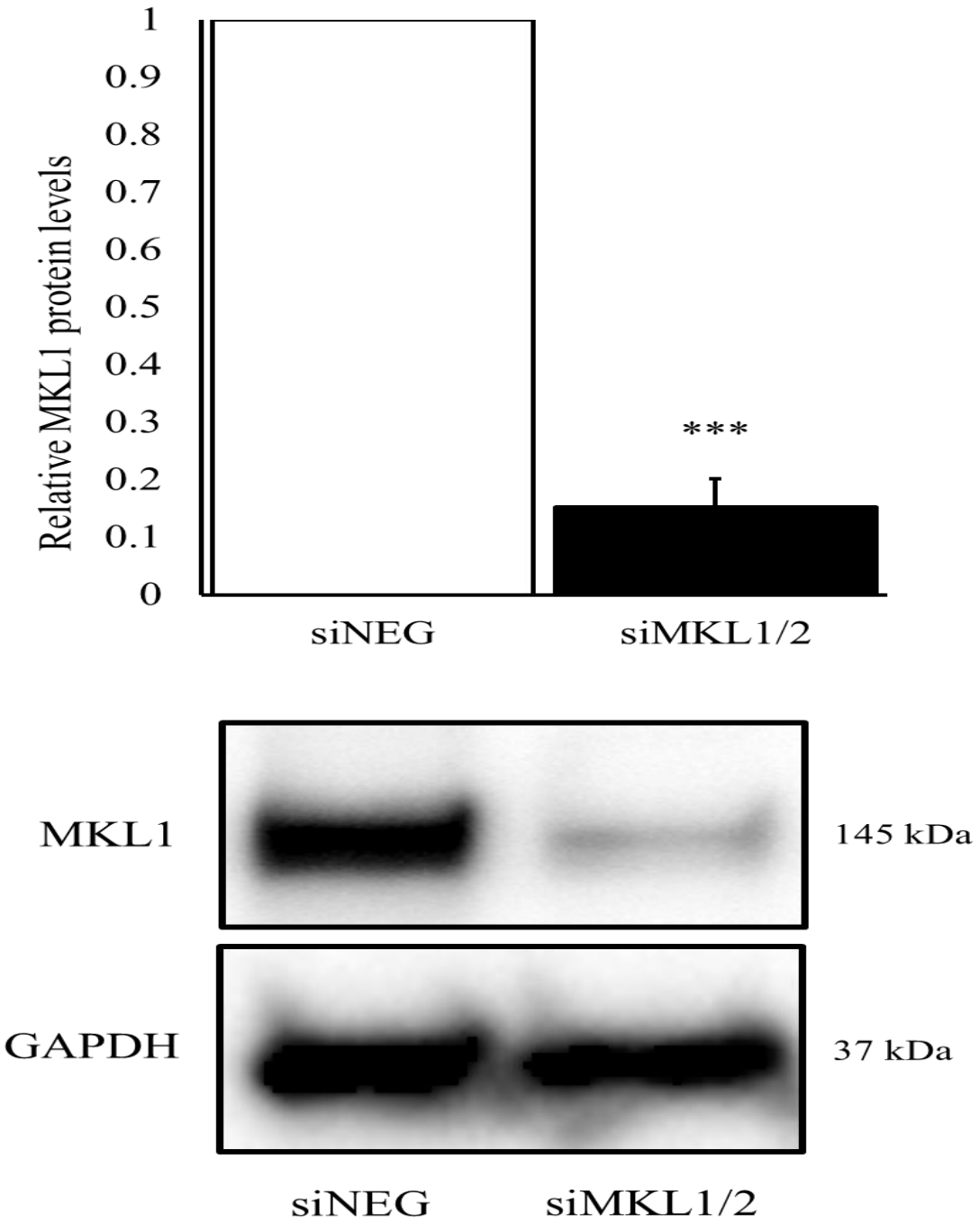


Figure 4.22: siMKL1/2 down regulates protein levels of MKL1 in cardiac fibroblasts

Rat cardiac fibroblasts were transfected with 50 pmoles of Silencer select siRNA targeting MKL1 and MKL2 (siMKL1/2) or non-targeting control siRNA (siNEG). Total cell lysates were prepared 48 hours post transfection and analysed by Western blotting for MKL1 and GAPDH. ***: $p<0.001$ and si: siRNA. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

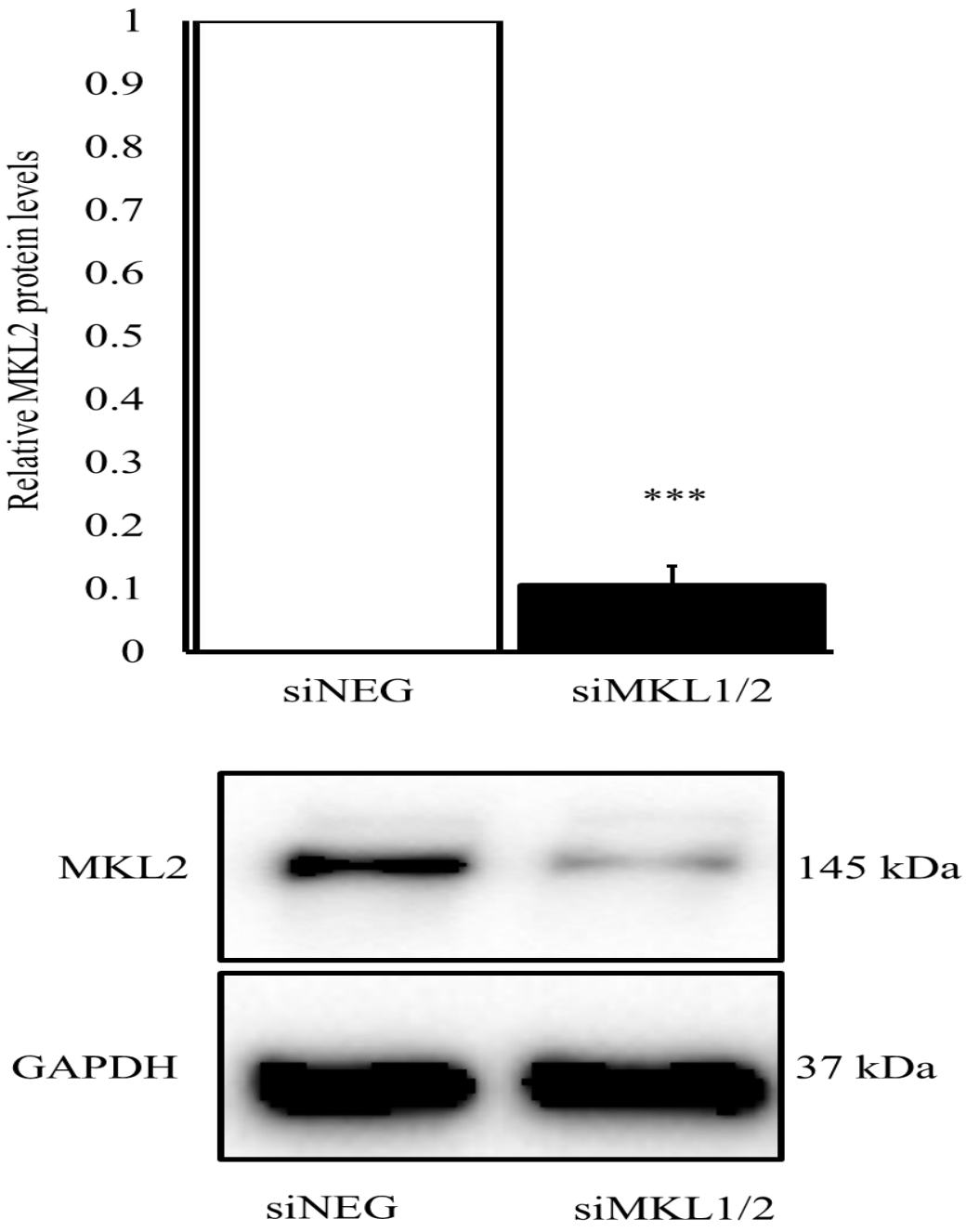


Figure 4.23: siMKL1/2 down regulates protein levels of MKL2 in cardiac fibroblasts.

Rat cardiac fibroblasts were transfected with 50 pmoles of Silencer select siRNA targeting MKL1 and MKL2 (siMKL1/2) simultaneously, or non-targeting control siRNA (siNEG). Total cell lysates were prepared 48 hours post transfection and analysed by western blotting for MKL2 and GAPDH. ***: $p<0.001$ and si: siRNA. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

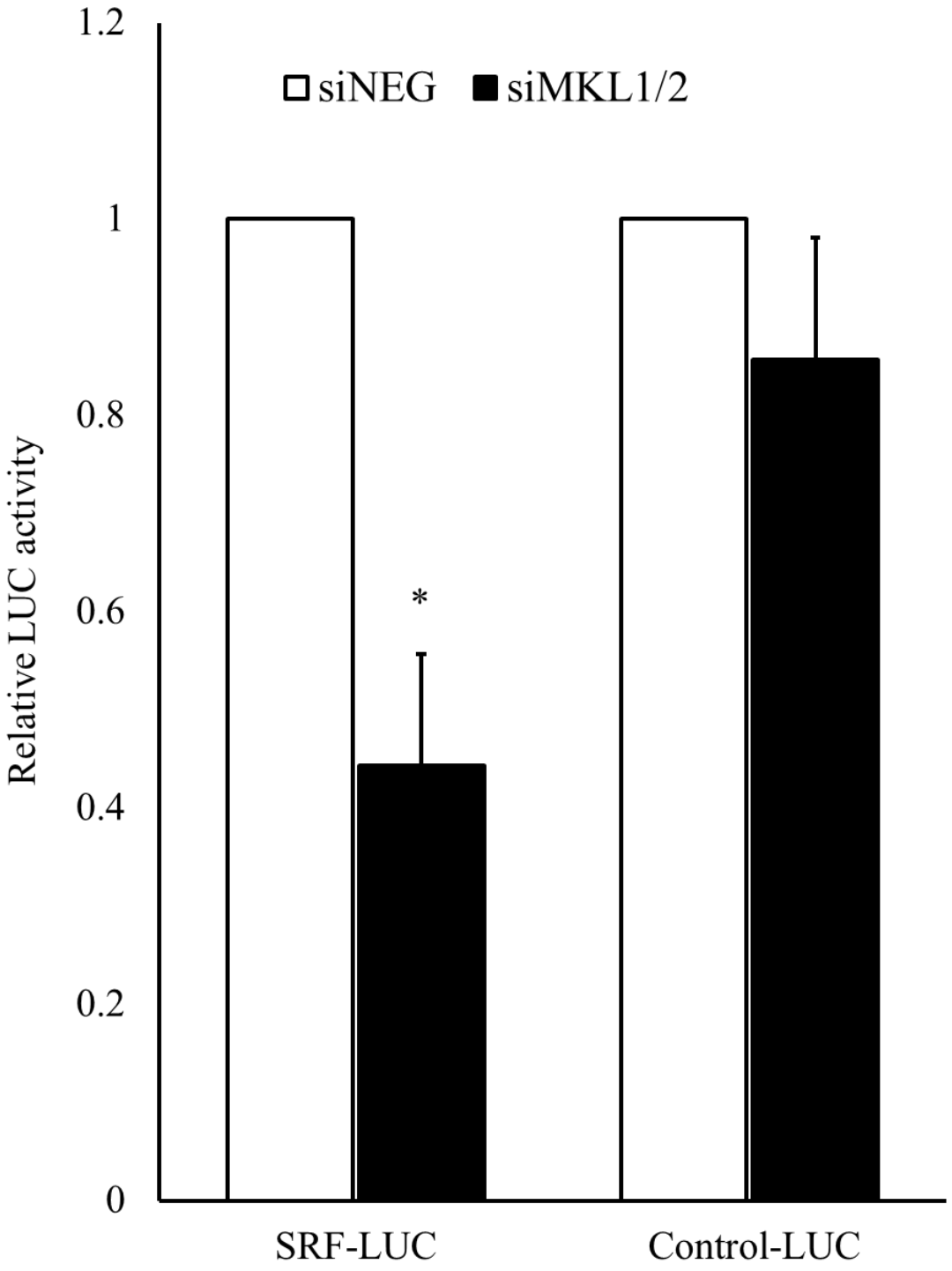


Figure 4.24: siMKL1/2 down regulate the activity of SRF promoter

Rat cardiac fibroblasts were transfected with 50 pmoles of Silencer select siRNA targeting MKL1 and MKL2 (siMKL1/2) simultaneously or non-targeting control siRNA (siNEG). Total cell lysates were analysed for reporter gene assay. *: $p<0.05$ and si: siRNA. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

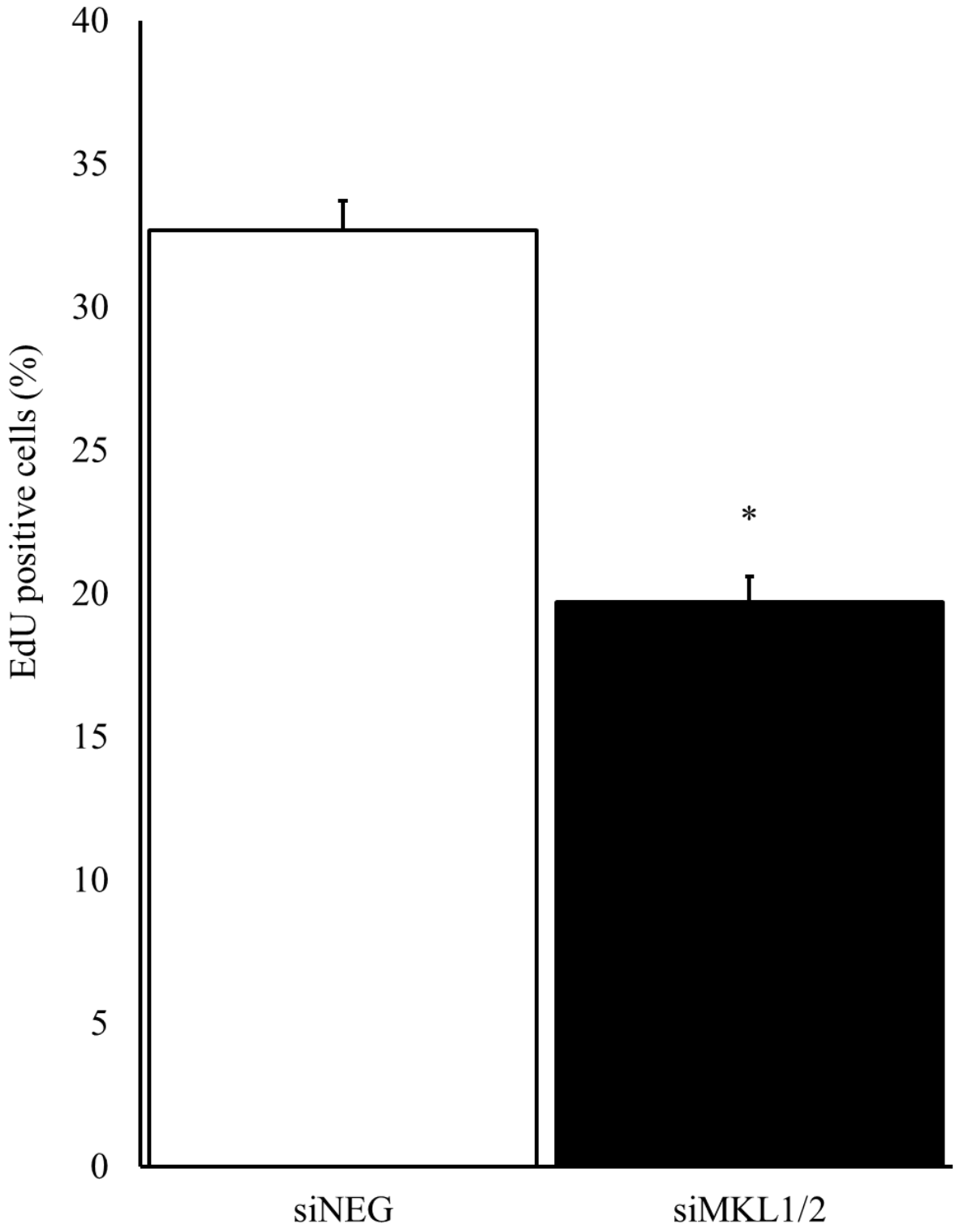


Figure 4.25: siMKL1/2 down regulates the proliferation of cardiac fibroblasts.

Rat cardiac fibroblasts were transfected with 50 pmoles of Silencer select siRNA siRNA targeting MKL1 and MKL2 (siMKL1/2) simultaneously or non-targeting control siRNA (siNEG). Cells were labelled with EdU 48 hours after transfection. *: $p<0.05$ and si: siRNA. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

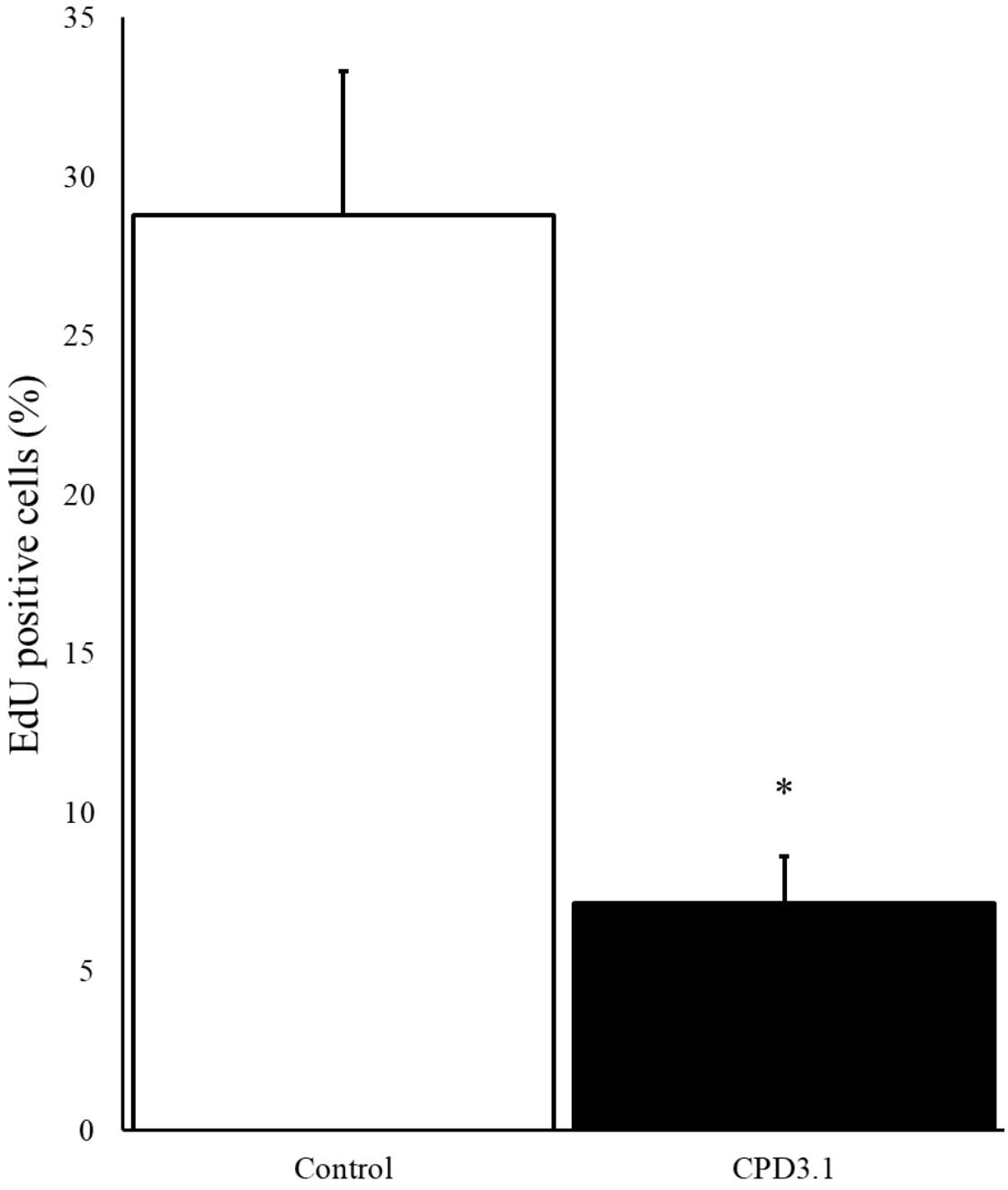


Figure 4.26: Compound 3.1 down regulates serum-stimulated proliferation of rat cardiac fibroblasts

Rat cardiac fibroblasts were stimulated for 24 hours with 60 µM CPD3.1 in 5% (v/v) serum, as indicated. Cells were labelled with 10 µM EdU for further 6 hours and proliferation quantified by immuno-histochemical staining of incorporated Edu. *: $p<0.05$ and CPD3.1: TEAD pharmacological inhibitor. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

4.4 DISCUSSION

Numerous investigations have characterised the ability of cAMP signalling to inhibit fibrosis (Kothapalli, Hayashi et al. 1998, Yokoyama, Patel et al. 2008, Insel, Murray et al. 2012, Simms, Coward et al. 2013, Weng, Wang et al. 2015, Liu, Xu et al. 2017, Lambers, Roth et al. 2018). In chapter 3, it was demonstrated that cAMP elevating stimuli inhibit the proliferation of cardiac fibroblasts without having significant effects on their migration. This anti-mitogenic effect was associated with a rapid and large change in cell morphology and a loss of actin stress fibres. Likewise, it was demonstrated that pharmacological ROCK inhibitor depolymerised the actin cytoskeleton of cardiac fibroblasts, down regulated their proliferation but increased their migration.

In chapter 4, the aforementioned findings were extended to characterise the mechanisms underlying the anti-mitogenic effects of cAMP signalling in cardiac fibroblasts. The data presented here demonstrates that elevated levels of cAMP inhibit the activity of two key transcription factors, known as, SRF and TEAD. Consistent with this, the mRNA levels of endogenous genes previously shown to be SRF and TEAD targets (Smith, Hudson et al. 2017, Smith, Sessions et al. 2019) were also down regulated by cAMP elevating stimuli. We also demonstrated that inhibition of SRF reporter gene activity is associated with nuclear exclusion of MKL1, an effect which was also regulated via PKA and EPAC stimulation. Moreover, we demonstrated that, stimulation of cAMP signalling in cardiac fibroblasts inhibited TEAD reporter gene activity by reducing the total protein levels of YAP and mediating its nuclear exclusion. Furthermore, work in this chapter highlight that of SRF and TEAD activities are functionally important in regulating the proliferation of cardiac fibroblasts.

As demonstrated in this chapter, cAMP elevating stimuli down regulate the activity of SRF and TEAD reporter genes. SRF and TEAD have previously been shown to be actin sensitive (Schratt, Philippar et al. 2002, Liu, Halayko et al. 2003, Miralles, Posern et al. 2003, Beck, Flynn et al. 2012, Oku, Nishiya et al. 2015, Kimura, Duggirala et al. 2016, Elisi, Santucci et al. 2018). Therefore, this is consistent with cAMP inhibition of these transcription factors, given that elevated levels of cAMP depolymerise actin filaments in fibroblasts and other cell types (Howe 2004, Pelletier, Julien et al. 2005, Gerits, Mikalsen et al. 2007, Kim, Kim et al. 2013, Chen, Yang et al. 2014, Kim, Ryu et al. 2015, Smith, Hudson et al. 2017). Although our data is consistent with this, further work is needed to demonstrate the essential role of the RhoA-ROCK pathway in mediating these inhibitory effects of cAMP on the activity of SRF and TEAD. This could be established using constitutively active mutants of RhoA to rescue the cAMP-induced inhibition of SRF and TEAD.

Moreover, we demonstrated the actin remodelling effects of cAMP is mediated by PKA and EPAC specific signalling. For example, we showed that selective PKA activation inhibited actin polymerisation and nuclear localisation of MKL1. Likewise, PKA and EPAC cooperate to induce morphological changes and inhibit nuclear localisation of MKL1. Interestingly, selective activation of EPAC also inhibited nuclear localisation of MKL1 in cardiac fibroblasts. To our knowledge, this is the first demonstration that EPAC regulates MKL1 localisation or SRF activity. Importantly, almost all studies link MKL1 regulation to remodelling of the actin cytoskeleton (Pawlowski, Rajakyla et al. 2010, Filippi 2015, Joy, Gau et al. 2017, Smith, Hudson et al. 2017, Sidorenko and Vartiainen 2019). However, we did not detect any morphological change in response to specific EPAC activation in cardiac fibroblasts. This may indicate the involvement of an actin-independent mechanism regulating nuclear localisation of MKL1 in response to EPAC signalling. However, this may simply reflect undetected or subtle changes in actin polymeristaion in response to EPAC activation that are regulating MKL localisation. Clearly, further research is required to investigate this further.

Consistent with previous findings, we showed that elevated levels of cAMP down regulated TEAD activity (Yu, Zhang et al. 2013, Varelas 2014, Meng, Moroishi et al. 2016). An effect which is mediated, at least in part, by translocation of YAP from the nucleus to the cytoplasm in cardiac fibroblasts. These data are consistent with those reported previously in VSMC. Since YAP cellular localisation is typically sensitive to the organisation of the actin cytoskeleton, our data would predict a cooperative role for both PKA and EPAC. However, this was not determined here. It will therefore be important to study the effects of selective PKA and EPAC activation on the protein levels and cellular localisation of total YAP, levels of phosphorylated YAP and TEAD activity.

SRF activity has a well characterised role in promoting the expression of immediate-early genes needed for proliferation and migration, such as, *FOS* (Treisman 1995). Also, it was shown that SRF activity in many adhesion dependent cell types, is dependent on RhoA signalling. For instance, expression of constitutively active RhoA mutants increase the polymerisation of actin (Liu, Halayko et al. 2003, Settleman 2003, Smith, Hudson et al. 2017, Gau and Roy 2018) and induce the expression of SRF-dependent genes, such as, *Ccn1*, *Ctgf* and *Pai1* (Smith, Hudson et al. 2017). In contrast, inhibition of RhoA with C3 transferase, inhibits the induction SRF-dependent genes by mitogenic stimuli (Hill, Wynne et al. 1995). Furthermore, stimulation of fibroblasts with agents that promote actin polymerisation (FCS and Jasplakinolide) or inhibit actin polymerisation (Lat-B and Cyto-D) demonstrate the dependency of SRF and TEAD activity on the organisation and polymerisation of the actin cytoskeleton (Sotiropoulos, Gineitis et al. 1999, Olson and Nordheim

2010, Gualdrini, Esnault et al. 2016, Smith, Hudson et al. 2017). Several lines of evidence also demonstrated the important roles of MKL1/2 in regulating proliferation (Seifert and Posern 2017, Gau and Roy 2018, Guenther, Faisal et al. 2019). Consistent with these findings, our data demonstrated that elevated levels of cAMP, down regulated RhoA-ROCK signalling, induced actin depolymerisation, suppressed SRF activity, inhibited nuclear localisation of MKL1 and proliferation. This implies that some of the anti-fibrotic effects of cAMP signalling pathway could be mediated by the inhibition of MKL signalling in cardiac fibroblasts. A number of published investigations support the statement that MKL signalling is pro-fibrotic (Parmacek 2010, Sakai, Chun et al. 2013, Shiwen, Stratton et al. 2015). For instance, MKL1 was demonstrated to have crucial roles in systemic sclerosis fibrosis and inhibition of MKL1 nuclear localisation or knockdown of MKL1 synthesis reduced collagen I production and abolished fibrotic targets in skin fibrosis (Shiwen, Stratton et al. 2015). In addition, investigations in mice reported the pro-fibrotic role of MKL1 in kidneys and showed that MKL1-deficient mice demonstrated significantly lower tubulointerstitial fibrosis compared to the control group (Xu, Wu et al. 2015). Moreover, studies in lung fibroblasts demonstrated that knocking down MKL1 reduced collagen synthesis and myofibroblasts transformation (Ni, Dong et al. 2013). Interestingly, Small and colleagues reported important new findings demonstrating that MKL1 plays central roles in promoting cardiac fibrosis in response to MI and MKL1 knockout mice demonstrated a significant reduction in the conversion of cardiac fibroblasts to myofibroblasts and cardiac fibrosis (Small, Thatcher et al. 2010). Consistent with these findings, our results suggest that anti-fibrotic effects of cAMP are at least in part mediated via the inhibition of MKL and that pharmacological targeting of MKL therapeutic potential for cardiac fibrosis.

Along these lines, pharmacological MKL inhibitors have been shown to have anti-fibrotic effects. For example, pharmacological inhibition of MKL reduced peritoneal fibrosis in mice (Sakai, Chun et al. 2013), down regulated TGF-β induced-fibrogenesis in human induced colonic myofibroblasts (Johnson, Rodansky et al. 2014) and decreased lung fibrosis in murine models (Sisson, Ajayi et al. 2015). The MKL inhibitor CCG1423 has been demonstrated to have anti-fibrotic effects (Minami, Kuwahara et al. 2012, Sakai, Chun et al. 2013). However, this first generation of MKL inhibitor has been reported to have some cytotoxic effects *in vitro* and *in vivo* (Evelyn, Bell et al. 2010, Bell, Haak et al. 2013, Johnson, Rodansky et al. 2014). Therefore, we assayed the anti-fibrotic effects of CCG203971, an optimised version of the previous generation (CCG1423), which was characterised by high throughput screening as an effective inhibitor of RhoA-stimulated SRF/MKL gene transcription (Bell, Haak et al. 2013). We demonstrated that CCG203971 significantly down

regulated the proliferation of cardiac fibroblasts, implicating MKL activity in the regulation of cardiac fibroblast proliferation. However, data obtained using these inhibitors should be interpreted cautiously as their exact mechanism of action is not fully understood. It has been suggested that they specifically binds to the NLS domains of MKL1/2, which prevents their interactions with importin α/β1 and inhibits their nuclear translocation (Hayashi, Watanabe et al. 2014). However, another investigation reported that these MKL pharmacological inhibitors interact with CasL (MICAL2), a member of MICAL family of actin binding monooxygenase (Lundquist, Storaska et al. 2014). Furthermore, MICAL2 is suggested to increase the activity of MKL via the polymerisation of nuclear actin (Grintsevich, Yesilyurt et al. 2016). As a result of this, some of the effects of CCG1423 on MKL1/2, could be due attributed to the inhibition of MICAL proteins (Haak, Appleton et al. 2017). Notably, even though CCG1423 and CCG203971 have been reported to down regulate MKL signalling pathway *in vitro* and *in vivo* (Haak, Tsou et al. 2014, Johnson, Rodansky et al. 2014, Sisson, Ajayi et al. 2015, Gau, Veon et al. 2017, Joy, Gau et al. 2017, Yu-Wai-Man, Spencer-Dene et al. 2017), these compounds are not only specific to MKL1/2, but rather inhibit all proteins containing the RPEL domain, for example: phosphatase and actin regulating proteins (PHACTR1), which is an actin binding protein with phosphatase regulating activity expressed in endothelial and vascular smooth muscle cells (Allen, Greenfield et al. 2004, Wiezlak, Diring et al. 2012, Huet, Rajakyla et al. 2013). The PHACTR1 gene is linked with different single nucleotide polymorphism which, have been associated with increased risk of heart diseases (Hager, Kamatani et al. 2012) and is expressed abundantly in human atherosclerosis plaques (Reschen, Lin et al. 2016). Since there are studies showing that PHACTR1 promote cellular migration and proliferation (Wiezlak, Diring et al. 2012, Fils-Aime, Dai et al. 2013, Jing, Zhang et al. 2019); therefore, it is important to note that, the inhibitory effects of CCG203791 on the proliferation of cardiac fibroblasts could be due to an accumulative result of global inhibition of proteins containing RPEL domains rather than MKL1 or MKL2 alone. However, the data presented here demonstrates that CCG203971 recapitulated the effects of cAMP signalling on the proliferation of cardiac fibroblasts.

To further test the role of MKL1/2 in cardiac fibroblast proliferation, we used siRNA-mediated gene silencing. Consistent with the result obtained from the pharmacological inhibition of MKL1/2, dual silencing of both MKL1 and MKL2 (siRNA MKL1/2) proteins significantly down regulated the proliferation of cardiac fibroblasts. These data strengthen the conclusion that MKL1/2 are important regulators of cardiac fibroblast proliferation and suggest that these may be involved in promoting cardiac fibrosis. Studies carried out in mice demonstrated that mice lacking MKL1 displayed reduced cardiac fibrosis and fewer SMA-positive myofibroblasts after MI (Small,

Thatcher et al. 2010). Importantly, mice lacking MKL1, did not suffer from cardiac rupturing following induced MI, indicating that they initially experienced sufficient scar formation to stabilise the injured myocardium (Small, Thatcher et al. 2010). Although we did not actually test the effects of siMKL1/2 on cardiac fibrosis, but we demonstrated that, siMKL1/2 reduced the proliferation of cardiac fibroblasts, implying lesser cardiac fibroblasts, lesser cardiac fibrotic genes expression and lesser ECM production, which is consistent with other studies, reporting the fibroblasts from mice lacking MKL1, displaying lower collagen synthesis (Luchsinger, Patenaude et al. 2011). MKL1 has also been recognised to have anti-apoptotic properties. Over expression of MKL1 has been reported to suppress tumour necrosis factor induced cell death in rat cardiomyocytes following hypoxia-induced injury (Zhong, Hu et al. 2016). The anti-apoptotic properties of MKL1 fits well with data establishing a role of MKL1 in the proliferation of cardiac fibroblasts.

Since MKL1 or MKL2 were not individually silenced in our experiments, the specific role of each MKL protein on the proliferation of cardiac fibroblasts could not be demonstrated. It is perhaps not surprising to expect divergent effects of MKL1 and MKL2 on the proliferation of cardiac fibroblasts, since MKL2 has a additional 71-amino acid N-terminal region that is not present in MKL1 and is suggested to act as an extra regulatory domain. Similar MKL1 and MKL1 gene silencing investigations from our group showed that MKL1 and MKL2 have divergent effects on the proliferation and migration of vascular smooth muscle cells (Smith, Hudson et al. 2017). It is therefore important that future research focuses on characterising the specific roles played by MKL1 and MKL2 in the regulation of cardiac fibroblast proliferation, migration and fibrosis.

Despite the advantages of silencing MKL1/2 over their pharmacological inhibitions, the findings presented here, could still be subject to off-target effects. Co-transcription factors such as: MKL1 and MKL2, are usually parts of a dynamic complex, with different interacting proteins that, incorporate intracellular and extracellular signals to direct gene expression accordingly. Silencing any of these proteins within their complexes creates the possibility to disassemble these complexes and inhibit the binding of other proteins. In fact, MKL1 and MKL2 have been demonstrated to directly interact with different proteins which regulate the transcription of SRF-target genes. For example, MKL1 and MKL2 have been reported to form homodimers and heterodimers with members of the myocardin transcription factor family via a coiled-core motif (Parmacek 2007). Indeed, it has been demonstrated that MKL1 binds to SRF in dimeric complexes (Miralles, Posern et al. 2003) and that disruption of this coiled-core domain in the structures of MKL1 and MKL2 down regulates the transactivation by these co-transcription factors (Du, Ip et al. 2003, Selvaraj and Prywes 2003). This highlights the significance of homodimerization and heterodimerisation for

their activities. In addition to these, several lines of evidence have reported the interaction of MKL1 with other transcription factors, for instance: STAT3 (Liao, Wang et al. 2014) and TEAD (Yu, Miyamoto et al. 2016). Out of importance, MKL1-STAT3 complexes have been demonstrated to enhance the migration of breast cancer cells (Liao, Wang et al. 2014) and the interaction of MKL1 with TEAD has been reported to be pro-mitogenic in human glioblastoma cells (Yu, Miyamoto et al. 2016). Furthermore, our group has also demonstrated that stimulation of the Hippo pathways in vascular smooth muscle cell increases proliferation (Kimura, Duggirala et al. 2016). Consequently, the effects on the proliferation of cardiac fibroblasts induced by MKL1/2 silencing could be a combined consequence of the removal of proteins and disruption of protein complexes that promote the proliferation of cardiac fibroblasts. Taken together, these observations suggest that the MKL-SRF and YAP-TEAD complexes may represent attractive drug targets to limit tissue fibrosis. However, it should be noted that a number of studies report possible limitations of MKL-SRF targeting therapies. For instance, general silencing of MKL2 induces embryonical lethality due to cardiac defects (Oh, Richardson et al. 2005) and specific silencing of MKL1/2 induced cardiac dilation, death of cardiomyocytes and even cardiac fibrosis in mice (Mokalled, Carroll et al. 2015).

Several lines of evidence report the roles of YAP/TAZ-TEAD in cellular proliferation (Camargo, Gokhale et al. 2007, Dong, Feldmann et al. 2007, Zhang, Smolen et al. 2008, Holden and Cunningham 2018). Also, the activities of YAP and TAZ were shown to be dependent on actin cytoskeleton and RhoGTPases (Dupont, Morsut et al. 2011, Aragona, Panciera et al. 2013, Reginensi, Scott et al. 2013). Consistent with these findings, our data demonstrated that elevated levels of cAMP, inhibited Rho-ROCK signalling, induced actin depolymerisation, suppressed nuclear localisation of YAP, decreased TEAD activity and proliferation. This implies that some of the anti-fibrotic effects of cAMP signalling pathway could be mediated at least in part via the inhibition of YAP/TEAD in cardiac fibroblasts. Accumulating evidence suggest that YAP/TAZ-TEAD signalling is pro-fibrotic (Piersma, Bank et al. 2015, Noguchi, Saito et al. 2018, Kim, Choi et al. 2019) and that they act as the sensors of ECM stiffness. The two transcriptional coactivators of Hippo pathway drive the synthesis of fibrogenic factors and increase cellular contractility mediating tissue stiffness mechanisms. As a result of this, YAP and TAZ stimulation could create a feed-forward loop of fibroblast activation which eventually would lead to tissue fibrosis (Noguchi, Saito et al. 2018). Interestingly, several studies demonstrated that YAP/TAZ-TEAD inhibition reduced fibrosis. For instance, the expression levels of YAP were increased in renal fibroblasts following kidney injuries (Liang, Yu et al. 2017). Moreover, inhibition of YAP/TAZ signalling pathway *in vitro* attenuated ECM synthesis and TGF-β induced-myofibroblast transformation,

whereas YAP over expression induced ECM synthesis and myofibroblast transformation even in the absence of TGF-β (Liang, Yu et al. 2017). Furthermore, it was shown that pharmacological inhibition of YAP (using verteporfin) or cell-specific knockout of YAP and TAZ reduced interstitial fibrosis in mice (Liang, Yu et al. 2017). Consistent with these findings, pharmacological inhibition of YAP and TAZ, using dopamine receptor agonist, reduced pulmonary and hepatic fibrosis in mice (Schnurrbusch, Jochmann et al. 2005). Taken together, these results highlight the importance of Hippo pathway signalling in fibrosis. However, it is important to consider the important adverse effects of verteporfin, such as: body pain, shortness of breath, chest pain and etc (Schnurrbusch, Jochmann et al. 2005). In addition, the endogenous levels of dopamine were not measured in liver and lung, therefore further investigations are needed to characterise the functional roles of dopamine signalling pathway during tissue fibrosis (Haak, Kostallari et al. 2019).

Consistent with a role of YAP/TAZ-TEAD in mediating cardiac fibroblast proliferation, we demonstrated, using a recently characterised novel TEAD inhibitor, CPD3.1 (Smith, Sessions et al. 2019), that pharmacological inhibition of TEAD using CPD3.1 reduced the proliferation of cardiac fibroblasts. Previously we demonstrated that CPD3.1 down regulated the activity of TEAD, CCN1 and CTGF promoter without affecting the control promoters, lacking the TEAD binding elements (Smith, Sessions et al. 2019). Moreover, we showed that, CPD3.1 down regulates the mRNA levels of TEAD target genes (CCN1 and CTGF) without affecting the mRNA levels of non-TEAD target genes (Smith, Sessions et al. 2019). All of which highlight the specificity of our novel TEAD inhibitor.

However, the precise role of YAP/TAZ-TEAD in cardiac fibrosis is still controversial. For example, genetic cardiac deletion of YAP resulted in increased cardiac fibrosis in response to pressure overload (Byun, Del Re et al. 2019). However, it is not clear if cardiac YAP knockout-induced cardiac fibrosis was due to an effect of YAP loss in cardiac fibroblasts or the loss of YAP in cardiomyocytes. It has been reported that YAP can act as a survival factor in cardiomyocytes and its loss results in increased cardiac myocytes apoptosis during pressure overload (Xin, Kim et al. 2013, Byun, Del Re et al. 2019). This increased cardiomyocyte death probably exacerbates the response to cardiac injury and in turn enhances the subsequent fibrotic response. Therefore, these findings imply that pharmacological inhibition of YAP may be problematic due to undesired effects of YAP inhibition in cardiomyocytes. Also, studies in rodents show that epicardial YAP and TAZ knockout induced cardiac fibrosis and death after MI in mice (Ramjee, Li et al. 2017), indicating the crucial roles of YAP/TAZ-TEAD for cardiac repair (Ramjee, Li et al. 2017, Liu and Martin 2019). In contrast, investigations in zebrafish and mice, demonstrated that YAP is activity is

required for scar formation rather than being required for cardiomyocyte proliferation, since its deletion was not sufficient to impair the proliferation of cardiomyocytes during cardiac regeneration (Flinn, Jeffery et al. 2019).

Importantly, four different isoforms of TEAD transcription factors (TEAD1-4) (Finch-Edmondson, Strauss et al. 2016) and multiple transcriptional coactivators (YAP, TAZ and VGLL) have been described that are likely to have distinctive functions in cardiac fibroblasts (Lin, Moroishi et al. 2018). It is therefore important that future research focusses on characterising the expression and function of these different TEADs and transcriptional coactivators in cardiac fibroblasts. This research may highlight the value of developing new TEAD isoform and/or co-factor specific inhibitors, which may provide anti-fibrotic effects without inducing unwanted side-effects such as increased cardiomyocyte death.

During tissue fibrosis, changes to the mechanical properties of the ECM have been implicated in promoting increased cardiac fibroblast proliferation and further enhancement of the fibrotic process. Targeting the activity of MKL-SRF and YAP/TAZ-TEAD may therefore represent a way to break this vicious cycle of fibrosis. This could initially be tested by culturing cardiac fibroblasts on bio-functionalised acrylamide gels of tuneable stiffness. Experiments using cultured myocardial slices could also explore this, with the advantage that cardiac fibroblasts are interacting with their endogenous natural ECM together with cardiac myocytes, which is clearly more physiologically relevant. Myocardial slices could provide representative *in vitro* models suitable model to investigate the proliferation (and possibly the migration) of cardiac fibroblasts in the context of cardiac fibrosis, as well as valuable tools to study the therapeutic interventions (Perbellini, Watson et al. 2018, Watson, Terracciano et al. 2019).

Taken together, the data presented in this chapter demonstrates that cAMP-mediated inhibition of cardiac fibroblast proliferation is mediated via reduced activity of both MKL-SRF and YAP-TEAD-dependent gene expression. This work implies that these transcription factor complexes may represent potential therapeutic targets to limit cardiac fibroblast proliferation during cardiac fibrosis. The data of this chapter is summarised in figure 4.27.

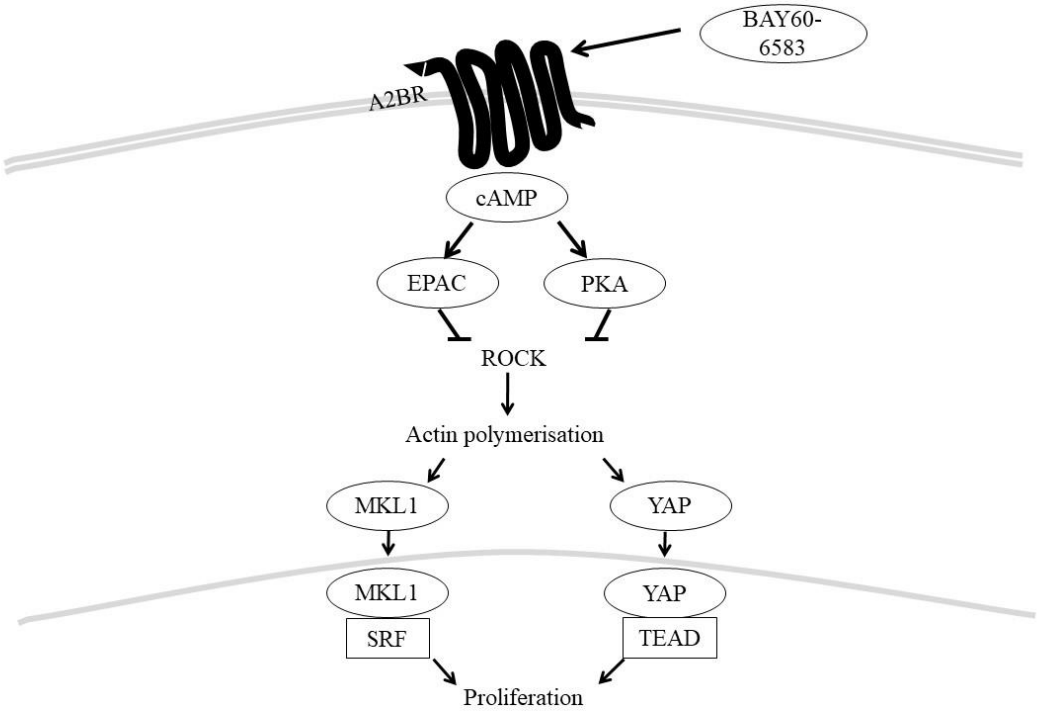


Figure 3.27. Schematic representation of the mechanism underlying the inhibition of cardiac fibroblast proliferation by adenosine induced cAMP signalling.

Adenosine signalling via the adenosine A2B receptor induces cAMP levels in cardiac fibroblast. This induces PKA and EPAC1 activity, which act cooperatively to inhibit actin polymeristaion. PKA and EPAC1 activation acts cooperatively to inhibit MKL1 and YAP nuclear localisation. Cyclic AMP signalling also induced YAP phosphorylation, a marker of YAP inactivation. Elevated cAMP levels inhibited the activity of the MKL1 and YAP-dependent transcription factors, SRF and TEAD, respectively. MKL1-SRF and YAP-TEAD activity are required for efficient proliferation in cardiac fibroblasts.

CHAPTER 5:

Elevated Cyclic-AMP represses expression of Exchange Protein Activated by cAMP (EPAC1) by inhibiting YAP-TEAD activity and HDAC-mediated histone deacetylation

5.1 INTRODUCTION

As mentioned in chapter 1, EPAC proteins are important cAMPs sensors that have been implicated in numerous physiological and pathological processes (Borland, Smith et al. 2009, Fujita, Umemura et al. 2017).

The development of EPAC-selective cAMP analogues such as 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP (Holz, Chepurny et al. 2008) has helped identify many important physiological functions of EPAC activity in cardiac cells. For example, EPAC activity has been implicated in the regulation of cardiac fibroblast proliferation (Phosri, Arieyawong et al. 2017), cell migration, contraction of cardiac myocytes (Pereira, Ruiz-Hurtado et al. 2012), regulation of inflammation (Parnell, Smith et al. 2012, Wiejak, Dunlop et al. 2014), cardiac myocyte hypertrophy (Metrich, Lucas et al. 2008) and cardiac fibrosis (Surinkaew, Aflaki et al. 2019). Although two isoforms of EPAC have been identified, namely EPAC1 and EPAC2, EPAC1 is more widely expressed than EPAC2, being ubiquitously expressed in cardiac cells (Ulucan, Wang et al. 2007, Olmedo, Munoz et al. 2013).

Cardiac fibroblasts represent the main non-myocyte cell type in the myocardium (Liu, Wang et al. 2017). They occupy the myocardial interstitium (Chen, Ding et al. 2013), performing various important functions that are required for maintenance of normal myocardial homeostasis, including regulation of myocardial ECM production and myocardial integrity in addition to acting as mechano-electric transducers (Souders, Bowers et al. 2009). In response to a range of different signals, including angiotensin II and TGF-β, cardiac fibroblasts trans-differentiate into myofibroblasts, characterised by increased expression of alpha-smooth muscle cell actin and increased proliferation, migration and collagen synthesis (Kong, Christia et al. 2014). The switch in phenotype from cardiac fibroblasts to myofibroblast is important for promoting myocardial healing after cardiac injury (Chistiakov, Orekhov et al. 2016, Shinde, Su et al. 2018). However, aberrant or excessive ECM deposition and myofibroblast proliferation has been implicated in the development of maladaptive fibrosis, which increases the stiffness of the myocardium, impairs cardiac function and promotes the development of heart failure (Kong, Christia et al. 2014, Medzikovic, de Vries et al. 2019).

As discussed in the introduction chapter, numerous reports have indicated that cAMP elevating stimuli, such as adenosine, have anti-fibrotic affects, which are believed to by mediated at least in part by reduced rates of cardiac fibroblast proliferation and a reduction in collagen production (Ryzhov, Sung et al. 2014, Phosri, Arieyawong et al. 2017, Vecchio, White et al. 2017). Importantly, activation of EPAC signalling has been implicated in these anti-fibrotic effects. Various *in vitro*

studies have reported that specific activation of EPAC1 using selective pharmacological agonists or genetic over expression, reduces angiotensin II-induced cardiac fibroblast proliferation, migration and collagen production (Yokoyama, Minamisawa et al. 2008, Villarreal, Epperson et al. 2009, Surinkaew, Aflaki et al. 2019) and reduces cardiac fibrosis *in vivo* (Surinkaew, Aflaki et al. 2019). In contrast, pharmacological inhibition of EPAC with ESI-09 reverses the inhibitory effects of adenosine on angiotensin II-induced collagen synthesis and cardiac fibroblast activation (Kong, Christia et al. 2014, Phosri, Bunrukchai et al. 2018). Taken together, these findings imply that activation of EPAC1 in response to elevated cAMP inhibits cardiac fibrosis.

Notably, several lines of evidence have suggested that levels of EPAC1 can be altered in response to different physiological and pathophysiological stimuli. For instance, growth factors, such as: angiotensin II and TGF-β enhance the reduction of EPAC1 mRNA and protein levels in cardiac fibroblasts (Yokoyama, Minamisawa et al. 2008, Olmedo, Munoz et al. 2013). EPAC1 mRNA and protein levels are also downregulated *in vivo* in a rat MI model and a canine ventricular tachypacing model of heart failure (Yokoyama, Minamisawa et al. 2008, Surinkaew, Aflaki et al. 2019).

Although a large body of literature documents the activation of EPAC GEF activity in response to cAMP, very little is known about the mechanisms that regulate EPAC1 expression levels. Since many cell signalling pathways are regulated by negative feedback loops that ensure transient signalling in response to specific stimuli, we hypothesised that EPAC1 expression may be subject to negative regulation in response to elevated cAMP levels. Here we characterise the regulation of EPAC1 gene expression in response to cAMP elevation in cardiac fibroblasts. We describe a novel negative feedback loop by which EPAC1 and PKA activation by cAMP reduces EPAC1 expression by inhibiting TEAD activity and histone-H3 lysine-27-acetylation of the EPAC1 proximal promoter.

5.2 HYPOTHESIS

The hypotheses for this chapter were:

1. Elevated cAMP inhibits EPAC1 expression in cardiac fibroblasts
2. EPAC1 expression is sensitive to cAMP induced actin remodelling.

5.3 RESULTS

5.3.1 Elevated cAMP inhibits EPAC1 expression in cardiac fibroblasts

Stimulation of A2BR increases cAMP levels and has anti-fibrotic effects in cardiac fibroblasts (Chen, Epperson et al. 2004). We therefore stimulated cardiac fibroblasts with the A2BAR agonist BAY60-6583 (at 5 µg/mL as previously described (Hudson, Kimura et al. 2018)) and quantified effects on EPAC1 mRNA and protein levels at early timepoints (i.e. less than 8 hours) to characterise direct effects on EPAC1 levels and avoid the possibility of any confounding effect of secondary signalling mechanisms. We focused on the regulation of EPAC1 levels as the expression of EPAC2 protein was not detectable in cultured rat cardiac fibroblasts by Western blotting (Figure 5.1), consistent with previous investigations (Yokoyama, Minamisawa et al. 2008).

Stimulation or rat cardiac fibroblasts with BAY60-6583 significantly reduced EPAC1 mRNA levels after 2 and 8 hours (Figure 5.2A). Levels of EPAC1 pre-spliced RNA, a measure of the transcription rate of EPAC1 (Elferink and Reiners 1996), were similarly reduced at 2 and 8 hours (Figure 5.2A). Levels of the housekeeping gene 36B4 remained unaffected. BAY60-6583 stimulation also reduced levels of EPAC1 protein after 2, 4, 6 and 8 hours, with maximal inhibition at the 8-hour time-point (Figure 5.3A). A similar down regulation of EPAC1 mRNA was detected in human cardiac fibroblasts, stimulated with BAY-60-6583 (Figure 5.4A).

We next tested if elevation of intracellular cAMP using the adenylate cyclase activator, forskolin (at 25 uM , as previously described (Smith, Hudson et al. 2017)) also inhibited EPAC1 expression. Stimulation with forskolin significantly inhibited EPAC1 mRNA and pre-spliced EPAC1 RNA at 2 and 8 hours without affecting 36B4 mRNA levels (Figure 5.2B). Forskolin stimulation also resulted in time dependent inhibition of EPAC1 protein levels, with maximal inhibition after 8 hours (Figure 5.3B). Forskolin stimulation of human cardiac fibroblasts with forskolin also resulted in the down regulation of both EPAC1 protein and mRNA levels, after 8 hours (Figure 5.4B and 5.5).

Finally, we tested if stimulation with the synthetic cAMP analogue, dibutyryl-cAMP (at 200 µM as previously described (Hewer, Sala-Newby et al. 2011)), also repressed EPAC1 expression. Stimulation of cardiac fibroblasts with dibutyryl-cAMP significantly suppressed EPAC1 mRNA and pre-spliced EPAC1 mRNA at 2 and 8 hours (Figure 5.2C) and EPAC1 protein levels (Figure 5.3C).

Elevated cAMP can activate both PKA and EPAC signalling. We therefore characterised if PKA and/or EPAC activity was involved in the inhibition of EPAC1 expression in cardiac fibroblasts. We initially used the PKA and EPAC selective pharmacological inhibitors (H89 and ESI-09

respectively) (Almahariq, Mei et al. 2014). Incubation of cardiac fibroblasts with H89 or ESI-09 significantly reversed the forskolin-induced reduction in EPAC1 mRNA and pre-spliced EPAC1 mRNA levels (Figure 5.6A). The mRNA levels of 36B4 were not altered (Figure 5.6A). These data imply the contribution of both PKA and EPAC activity in the regulation of EPAC1 mRNA levels.

In order to test the roles of PKA and EPAC activation further, cardiac fibroblasts were stimulated with the PKA selective agonist 6-BNZ-cAMP-AM or the non-cyclic EPAC selective agonist I-942 (Parnell, McElroy et al. 2017). Stimulation with 6-BNZ-cAMP-AM or I-942 alone induced a significant reduction on the mRNA levels of EPAC1 and pre-spliced EPAC mRNA without affecting levels of 36B4 (Figure 5.6B). Importantly, co-stimulation of cardiac fibroblasts with I-942 and 6-BNZ-cAMP-AM acted additively to downregulate EPAC1 mRNA and pre-spliced RNA levels (Figure 5.6B).

To further test the role of EPAC activation in the regulation of EPAC1 mRNA levels, we stimulated cells with the cell permeable EPAC selective cAMP analogue 8-pCPT-2'-O-Me-cAMP-AM (abbreviated to 8-CPT-cAMP-AM) alone or with the PKA selective agonist 6-BNZ-cAMP-AM.

Specific activation of EPAC with 8-pCPT-2'-O-Me-cAMP-AM or PKA with 6-BNZ-cAMP-AM alone significantly suppressed EPAC1 mRNA and pre-spliced EPAC mRNA without changing the mRNA levels of housekeeping genes (Figure 5.7A). A combination of 6-BNZ-cAMP-AM plus 8-pCPT-2'-O-Me-cAMP-AM acted additively to suppress EPAC1 mRNA and pre-spliced RNA (Figure 5.7A). Likewise, the mRNA levels of 36B4 were unaffected (Figure 5.7A).

In addition to the suppression of EPAC1 mRNA levels, EPAC1 protein levels were significantly inhibited in response to 6-BNZ-cAMP-AM or a combination of 6-BNZ-cAMP-AM with 8- cAMP-AM but not by 8-CPT-cAMP-AM alone (Figure 5.7B).

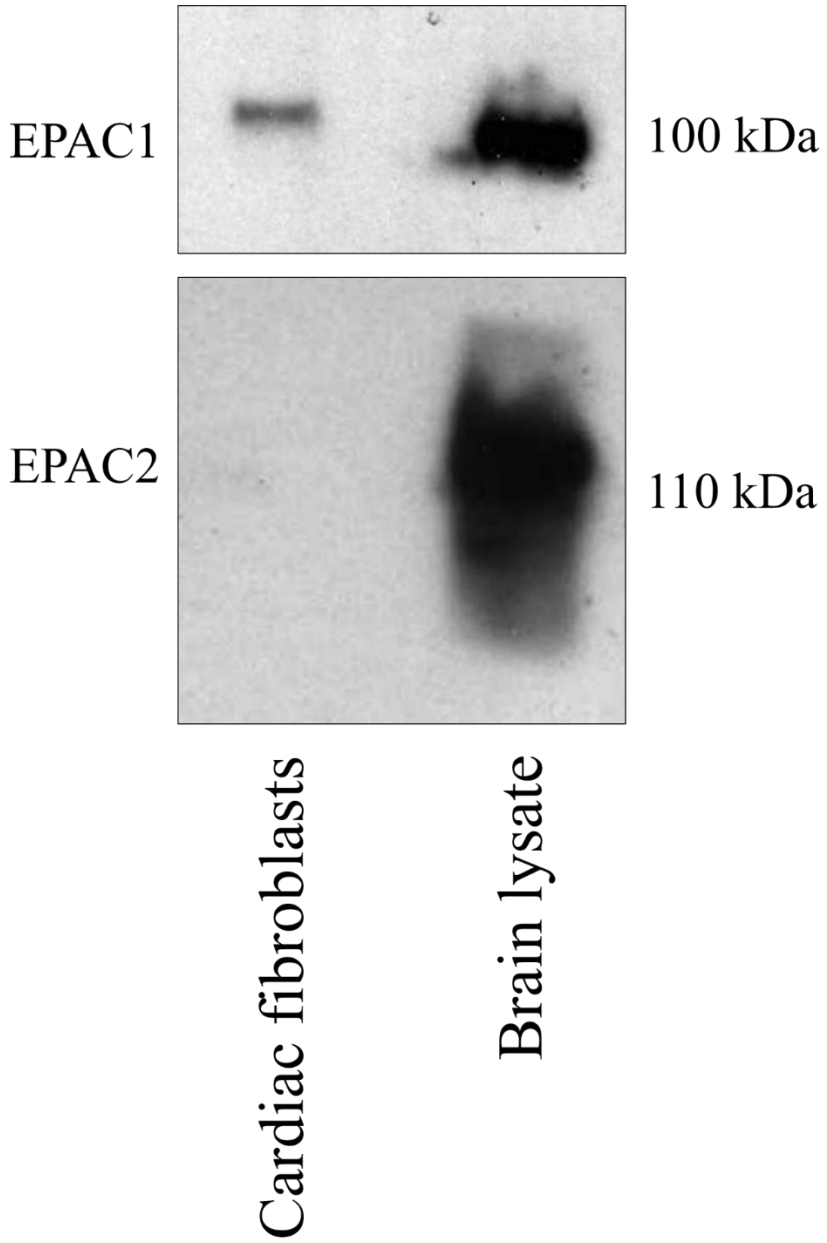


Figure 5.1: Analysis of EPAC1 and EPAC2 protein levels in cardiac fibroblasts

Total cell lysates prepared from rat cardiac fibroblasts were analysed for EPAC1 and EPAC2 protein levels by Western blotting. Rat brain lysate was used as a positive control for EPAC1 and EPAC2.

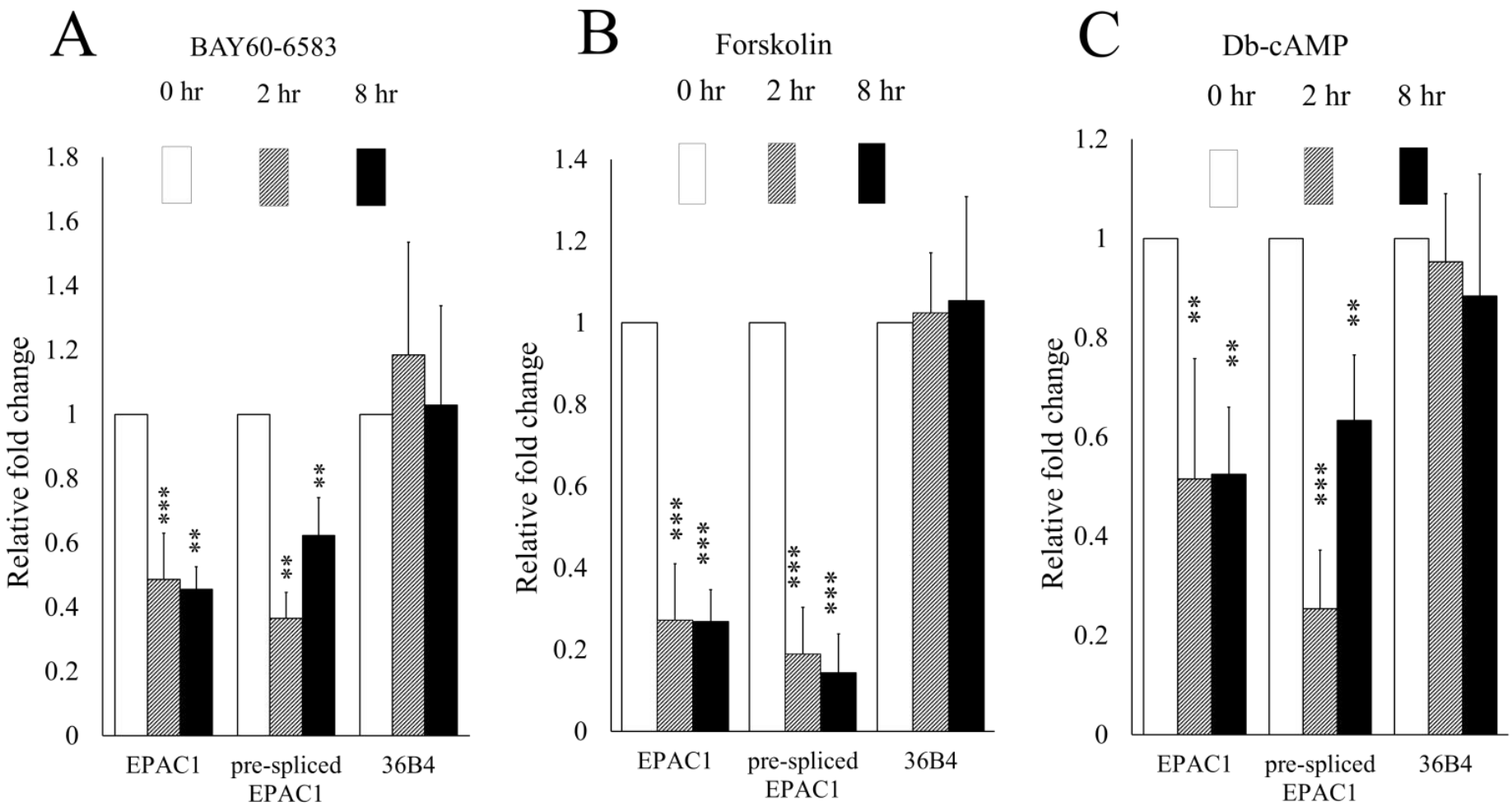

A
BAY60-6583
0 hr
2 hr
8 hr
Relative fold change
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
**
**
**
EPAC1
pre-spliced EPAC1
36B4
B
Forskolin
0 hr
2 hr
8 hr
Relative fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
***
EPAC1
pre-spliced EPAC1
36B4
C
Db-cAMP
0 hr
2 hr
8 hr
Relative fold change
1.2
1
0.8
0.6
0.4
0.2
0
**
**
***
**
EPAC1
pre-spliced EPAC1
36B4

Figure 5.2: cAMP elevating stimuli down regulate EPAC1 mRNA levels in cardiac fibroblasts

Serum-starved rat cardiac fibroblasts were stimulated with BAY60-6583 (5 µg/mL; **A**), forskolin (25 µM; **B**) and dibutyryl-cAMP (200 µM; **C**) for 2 to 8 hours. Total RNA was extracted and analysed by qRT-PCR for fully spliced and pre-spliced EPAC1 and 36B4 mRNA (**A**, **B** and **C**). **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

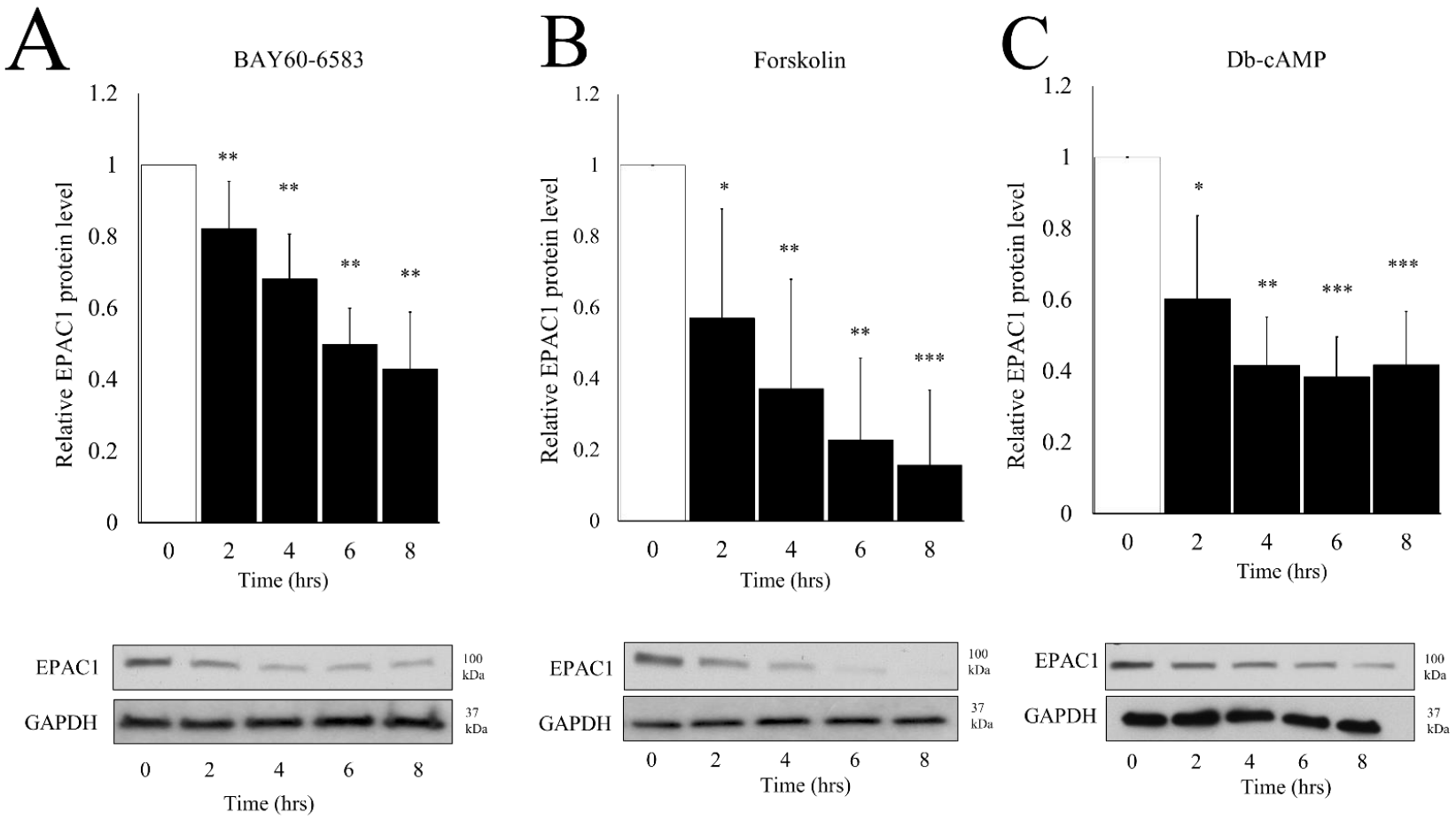

A
BAY60-6583
Relative EPAC1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
**
0
2
4
6
8
Time (hrs)
EPAC1
GAPDH
100 kDa
37 kDa
0
2
4
6
8
Time (hrs)
B
Forskolin
Relative EPAC1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
***
0
2
4
6
8
Time (hrs)
EPAC1
GAPDH
100 kDa
37 kDa
0
2
4
6
8
Time (hrs)
C
Db-cAMP
Relative EPAC1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
*
**
***
***
0
2
4
6
8
Time (hrs)
EPAC1
GAPDH
100 kDa
37 kDa
0
2
4
6
8
Time (hrs)

Figure 5.3: cAMP elevating stimuli down regulate EPAC1 protein levels in cardiac fibroblasts

Serum-starved rat cardiac fibroblasts were stimulated with BAY60-6583 (5 µg/mL; **A**), forskolin (25 µM; **B**) and dibutyryl-cAMP (200 µM; **C**) for 2, 4, 6 and 8 hours. Total cell lysates were analysed for the levels of EPAC1 and GAPDH protein by Western blotting and densitometric analysis (**A**, **B** and **C**). *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed using one-way ANOVA with Student Newman-Keuls post-test, n=3.

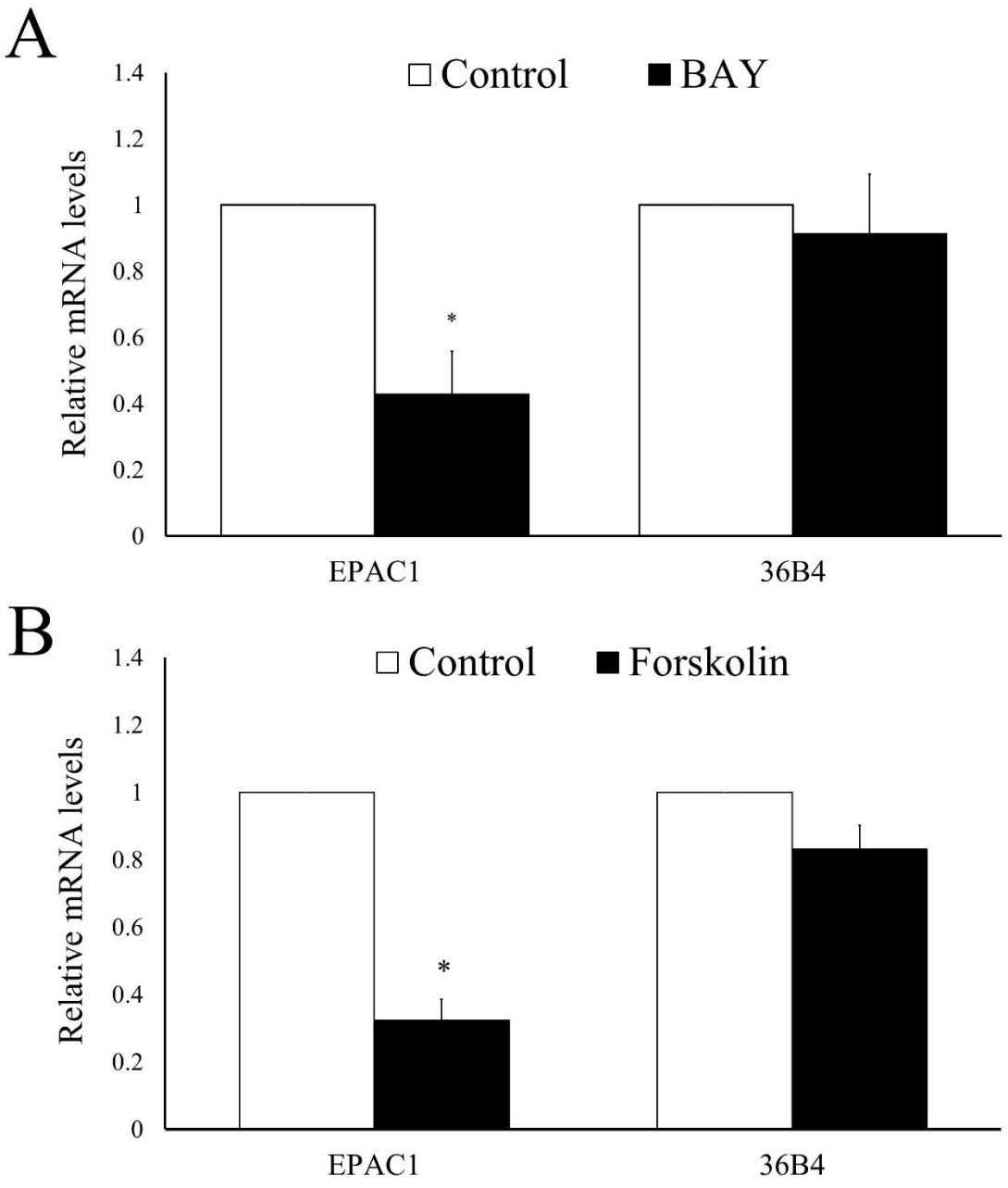


Figure 5.4: A2BR activation and forskolin stimulation inhibit EPAC1 mRNA in human cardiac fibroblasts

Human cardiac fibroblasts were stimulated with BAY60-6583 (5 µg/mL; **A**) or forskolin (25 µM; **B**) for 8 hours. Total RNA was extracted and analysed by qRT-PCR for EPAC1 and 36B4 mRNA. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=5.

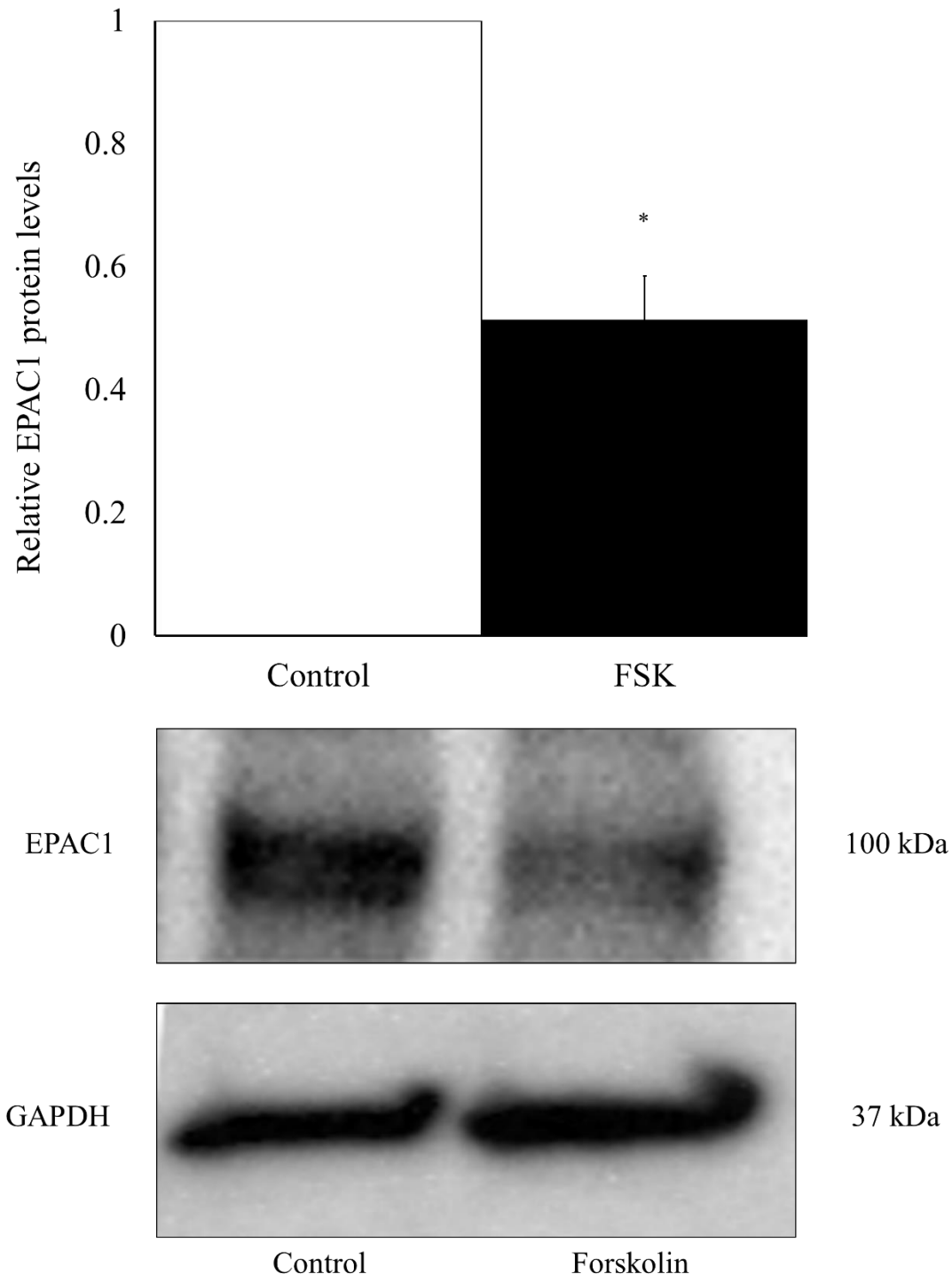


Figure 5.5: Forskolin stimulation inhibits EPAC1 protein levels in human cardiac fibroblasts

Human cardiac fibroblasts were stimulated with 25 µm forskolin for 8 hours. Total cell lysates were analysed for the levels of EPAC1 and GAPDH protein by Western blotting and densitometric analysis. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=4.

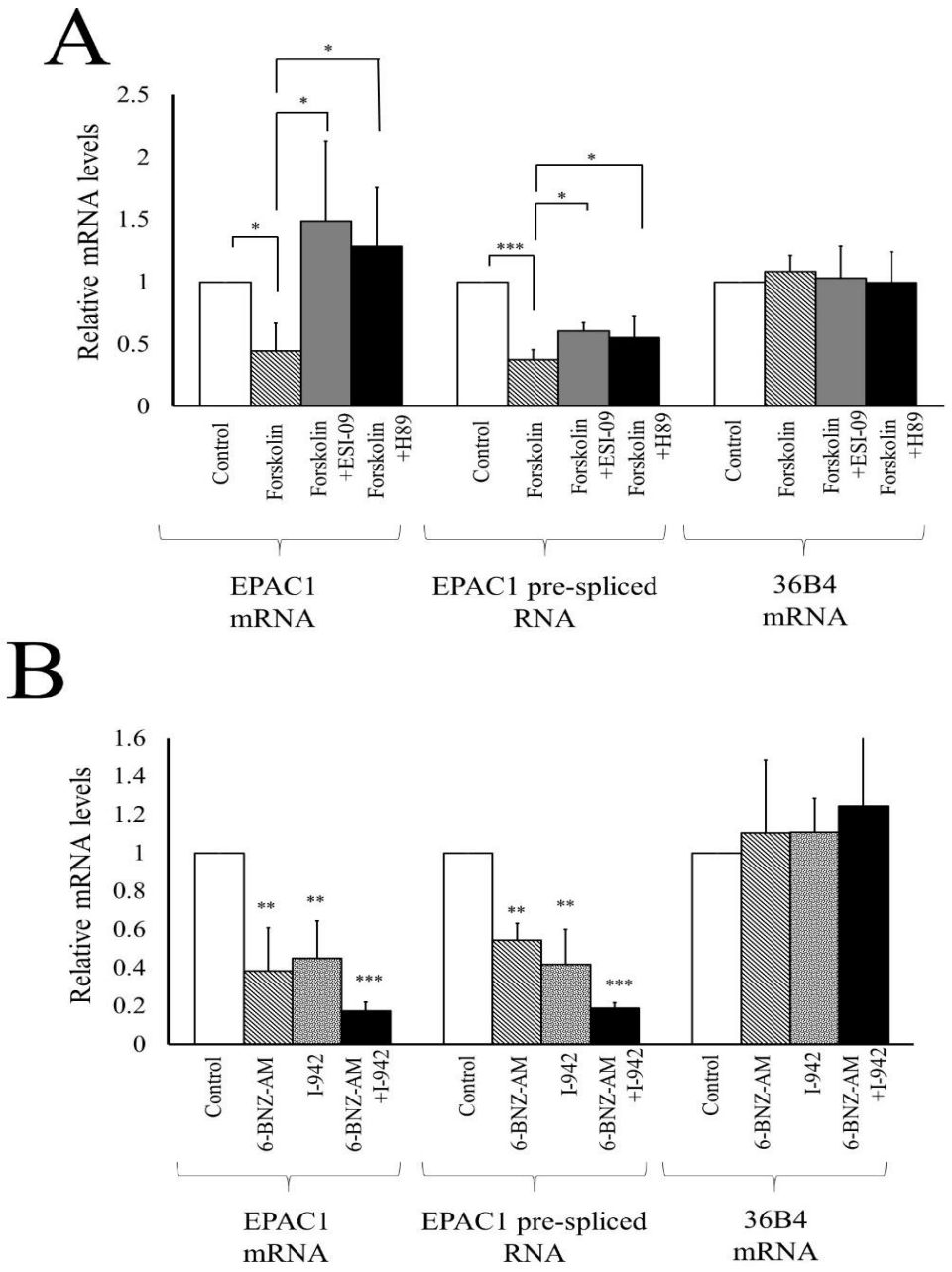


Figure 5.6: PKA and EPAC activity co-ordinately regulate EPAC1 expression in cardiac fibroblasts

Serum-starved CF were stimulated with forskolin (25 µM) alone or in combination with either H89 (10 µM) or ESI-09 (10 µM), as indicated, for two hours (**A**) or with 6-BNZ-cAMP-AM (6-BNZ-AM; 20 µM), I-942 (100 µM) or 8-pCPT-2'-O-Me-cAMP-AM (8-CPT-AM, 20 µM) as indicated, for 8 hours (**B**). Total RNA was extracted and analysed for EPAC1 mRNA, pre-spliced EPAC1 mRNA and 36B4 mRNA by qRT-PCR. *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, **A**, n=3 and **B**, n=5.

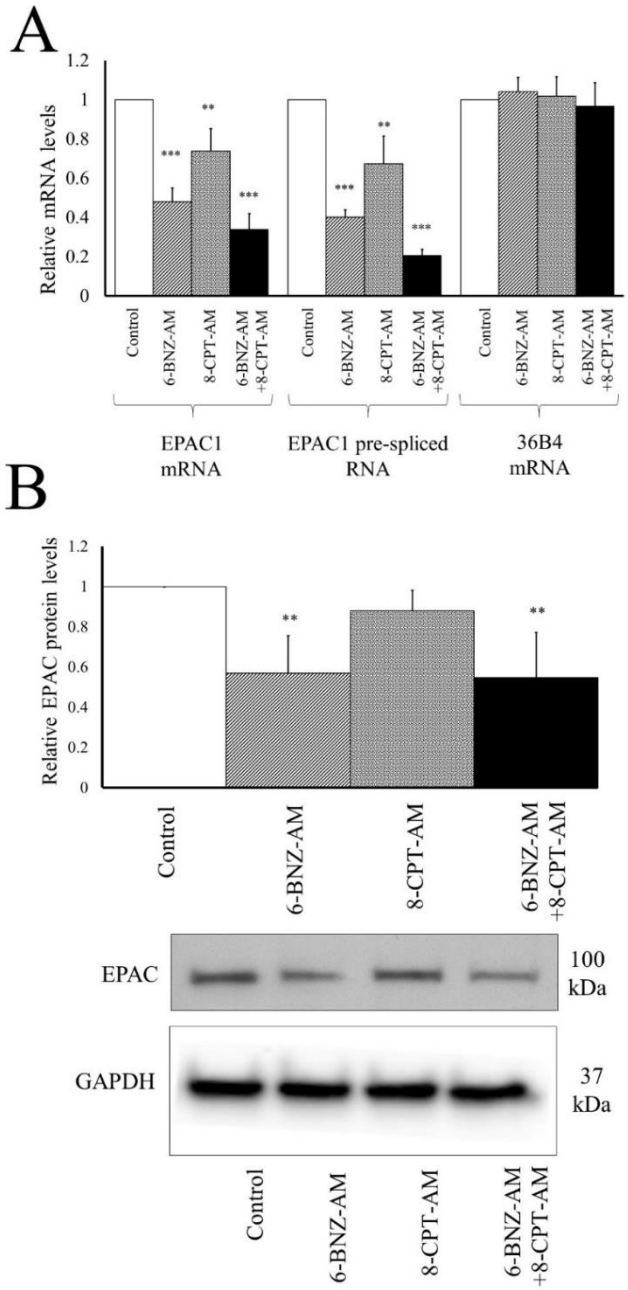


Figure 5.7: PKA and EPAC activity synergically downregulate EPAC1 expression in cardiac fibroblasts.

Serum-starved CF were stimulated with 6-BNZ-cAMP-AM (6-BNZ-AM; 20 µM), or 8-pCPT-2'-O-Me-cAMP-AM (8-CPT-AM, 20 µM) alone or in combination as indicated, for 8 hours. Total RNA was extracted and analysed for EPAC1 mRNA, pre-spliced EPAC1 mRNA and 36B4 mRNA by qRT-PCR (**A**). Total cell lysates were analysed for the levels of EPAC1 and GAPDH protein by western blotting and densitometric analysis (**B**). *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, **A**, n=3 and **B**, n=5.

5.3.2 Inhibition of EPAC1 expression by cAMP is associated with actin-cytoskeleton remodelling and changes in cell morphology

cAMP elevating stimuli have previously been documented to alter cell morphology and actin cytoskeleton remodelling in several cell types (Hewer, Sala-Newby et al. 2011, Duggirala, Kimura et al. 2015, Kimura, Duggirala et al. 2016, Smith, Hudson et al. 2017) and this has been associated with changes in cell behaviour. It was noticed that down regulation of EPAC1 protein and mRNA levels in response to cAMP-elevating stimuli was associated with this reduction in cell spreading (Figure 5.10A) and a change in cell morphology (Figure 3.4, 3.6, 3.8, 3.9 and 5.8), characterised by acquisition of stellate-like morphology, loss of F-actin-stress fibres and actin depolymerisation (Figure 5.9). We therefore tested if this change in cell morphology and disruption of actin polymerisation was functionally linked to changes in the levels of EPAC1 expression in cardiac fibroblasts. Importantly, cAMP elevation did not induce any detectable morphological change, reduction in cell area, or loss of F-actin stress fibres in the embryonic cardiomyocyte H9C2 cell line, Moreover, elevated cAMP in these cells did not result in any reduction in EPAC1 mRNA expression, suggesting a possible mechanistic link between cAMP induced actin remodelling and EPAC1 expression. in (Figure 5.8, 5.9, 5.10B and 5.11).

In order to further test the functional association between actin remodelling and EPAC1 expression in cardiac fibroblasts, we tested if removal of cAMP stimulus would result in a reversal of the cAMP stellate morphology and a restoration of EPAC1 mRNA expression levels back to normal control levels. Stimulating cardiac fibroblasts with forskolin for 2 hours, 8 hours or 24 hours significantly suppressed EPAC1 mRNA expression (Figure 5.12A) and induced a characteristic stellate morphology (Figure 5.12B). Notably, mRNA levels of EPAC1 after a 2-hour forskolin stimulation followed by a 6-hour or 24-hours washout period were not significantly different from baseline levels. This implies that cAMP washout leads to a rapid reversal of the forskolin-induced morphological change and restoration of EPAC1 expression (Figure 5.12).

The next question was whether actin polymerisation is necessary for maximal EPAC1 expression in cardiac fibroblast. In order to answer this question, actin cytoskeleton polymerisation was disrupted using multiple approaches, including incubation with the Rho kinase (ROCK) inhibitor Y27632, or the actin binding drugs Lat-B or Cyto-D. Treatment of cardiac fibroblasts with Y27632, Lat-B or Cyto-D rapidly induced stellate cell morphology and a loss of F-actin stress fibres (Figure 5.13A), which was morphologically similar to that induced by elevated cAMP. Likewise, treatment with these actin depolymerising agents significantly inhibited cell spreading (Figure 5.13B) and significantly inhibited EPAC1 mRNA and pre-spliced RNA levels, without affecting mRNA levels

of the housekeeping gene 36B4 (Figure 5.14A). Y27632, Lat-B or Cyto-D stimulation also significantly reduced levels of EPAC1 protein (Figure 5.14B). Taken together, these data demonstrate that EPAC1 expression is dependent on actin polymerisation.

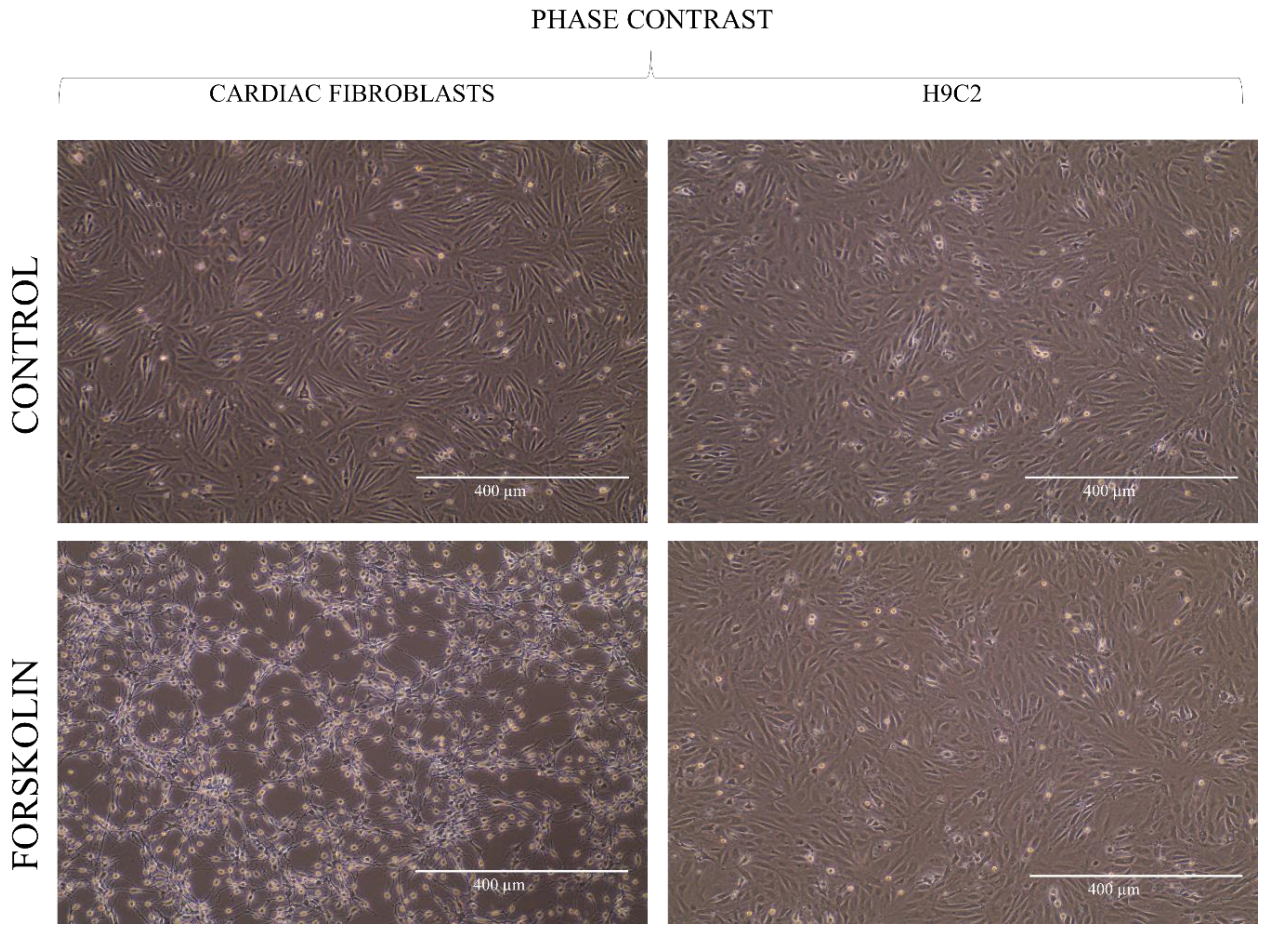


Figure 5.8. Forskolin induces morphological changes in cardiac fibroblasts but not in cardiac myocytes.

Rat cardiac fibroblasts and cardiac myocytes were stimulated in serum-free conditions with 25 µM forskolin and phase contrast microscopy taken after 8 hours.

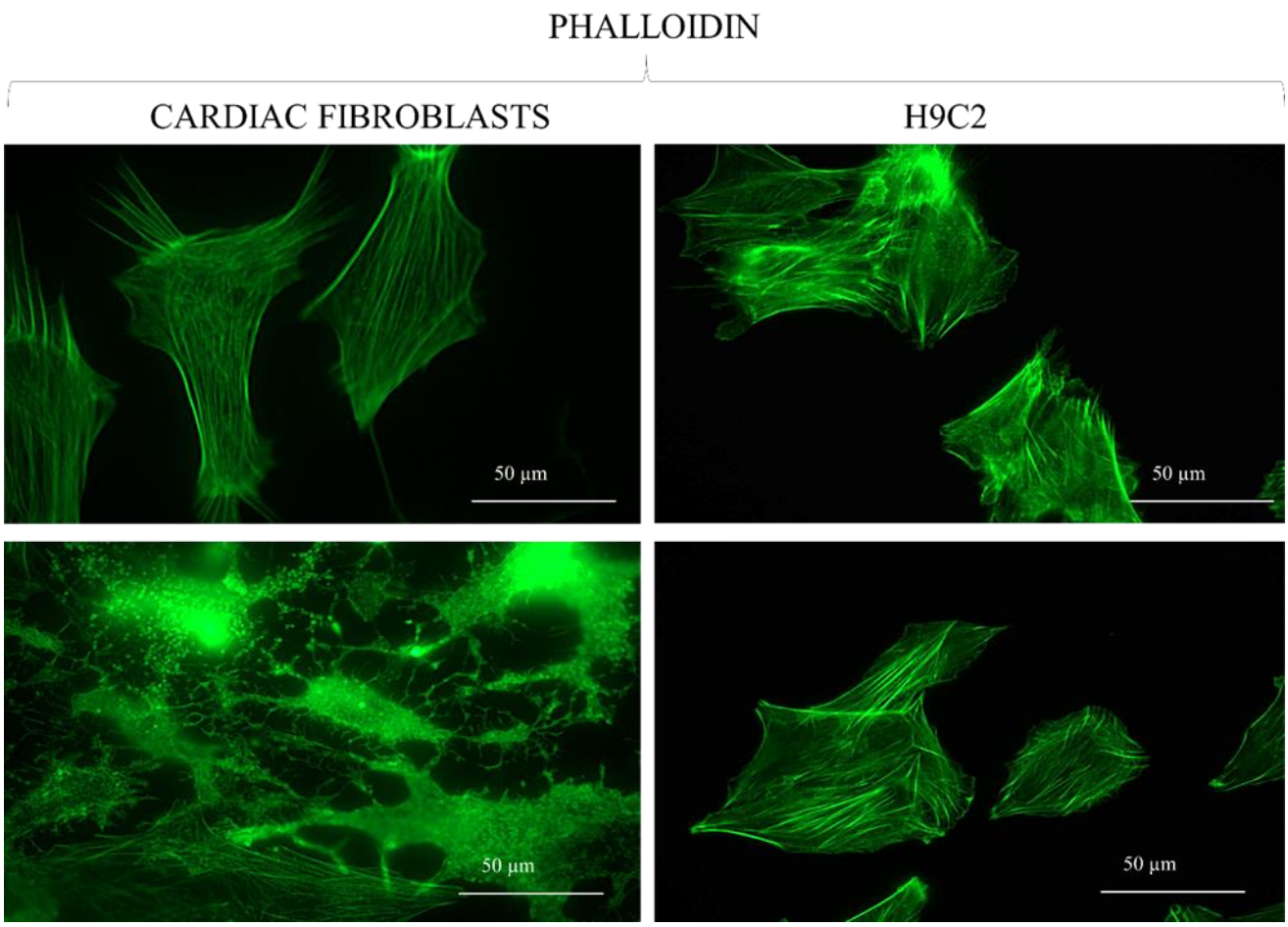


Figure 5.9 Forskolin stimulation disrupts the F-actin fibres in cardiac fibroblasts but not in cardiac myocytes.

Rat cardiac fibroblasts and cardiac myocytes were stimulated in serum-free conditions with 25 µM forskolin for 8 hours. Polymerised F-actin filaments were detected using Phalloidin staining of fixed cells. Representative images are shown.

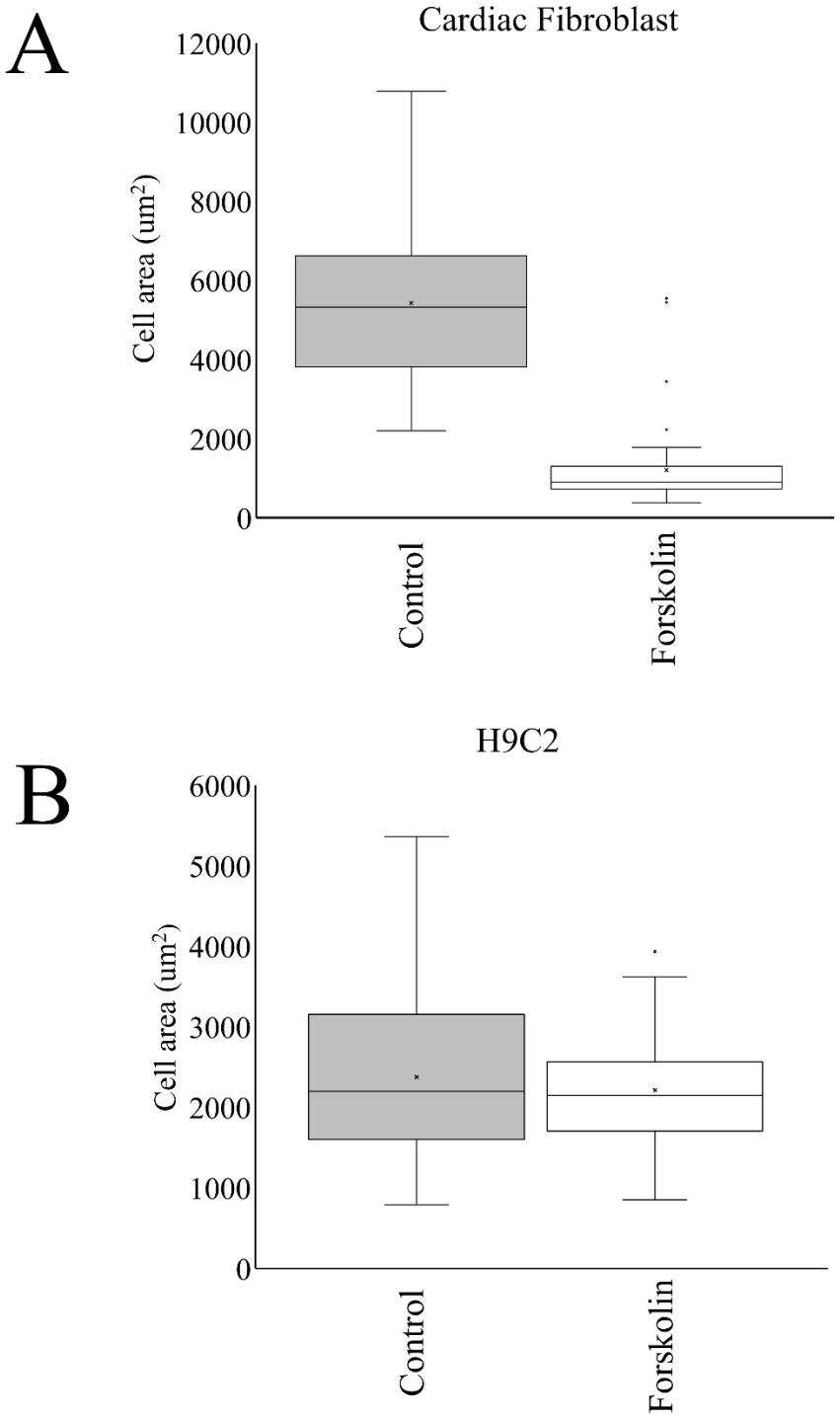


Figure 5.10. Forskolin stimulation inhibits cell spreading in cardiac fibroblasts but not H9C2 cells.

Serum-starved rat cardiac fibroblasts and H9C2 cells were stimulated with 25 µM forskolin for 8 hours. Cell spreading was analysed by phase contrast microscopy. *: $p<0.05$. Data are expressed as mean ±SEM and analysed using paired Students t-test, n=3.

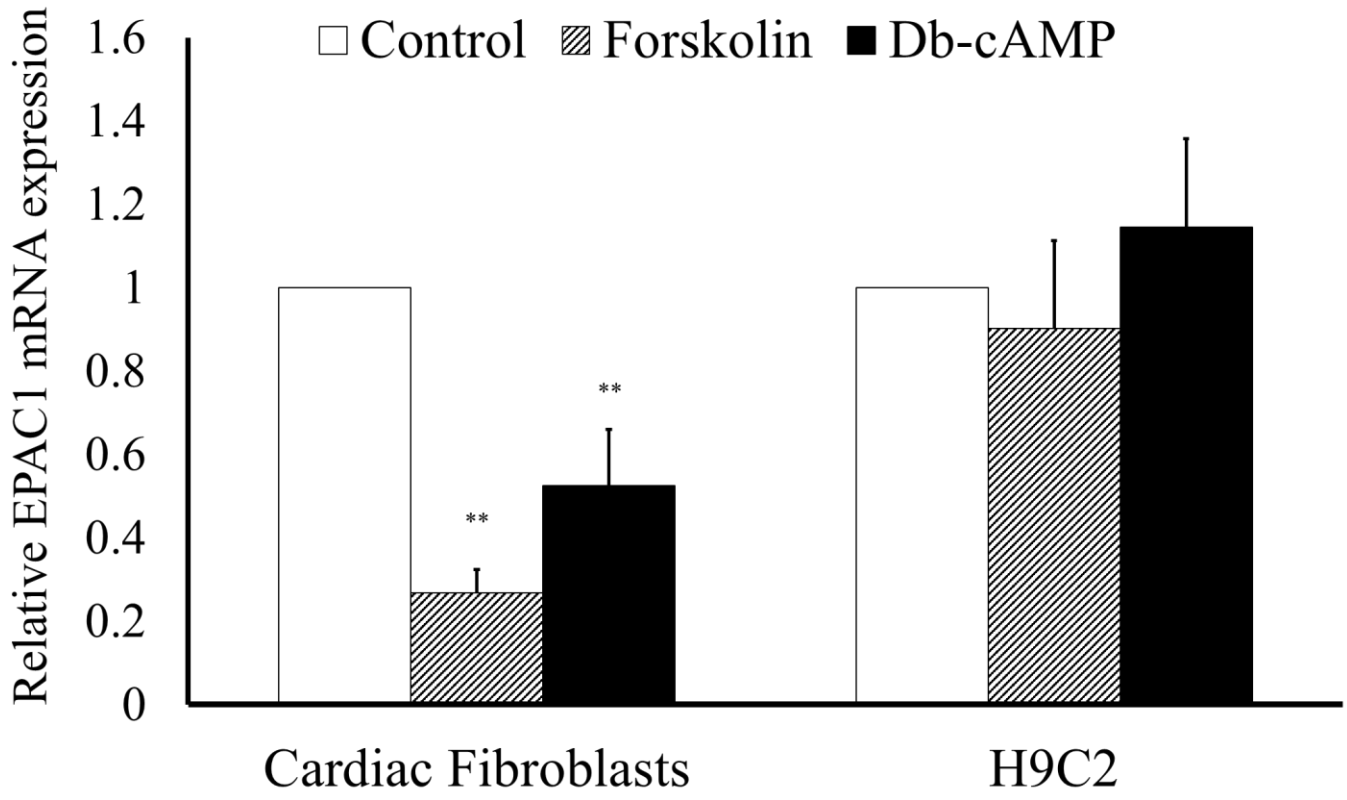


Figure 5.11. inhibition of EPAC1 expression by cAMP elevation.

Serum-starved rat cardiac fibroblasts and H9C2 cells were stimulated with 25 µM forskolin or 200 µM dibutyryl-cAMP for 8 hours, as indicated. Total RNA was extracted and analysed for EPAC1 mRNA by qRT-PCR. Db-cAMP analogue: Dibutyryl cAMP analogue and **: $p<0.01$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=3.

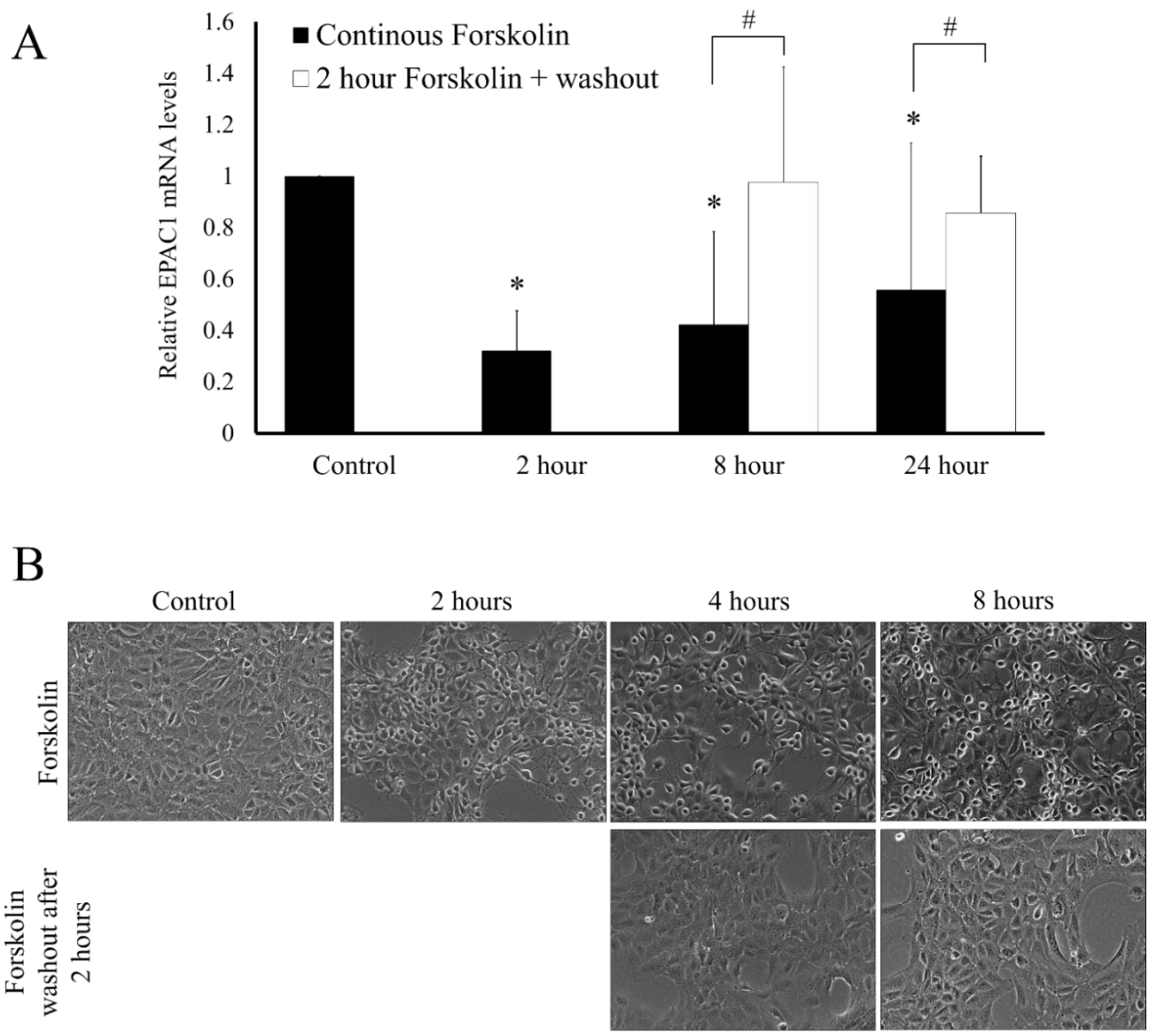


Figure 5.12. Washout of forskolin results in rapid reversal of stellate morphology and re-expression of EPAC1 mRNA.

Serum-starved rat cardiac fibroblasts were stimulated continuously with 25 µM forskolin for 2 hours, 8 or 24 hours. Cells were also stimulated with 25 µM forskolin for 2 hours followed by washout of forskolin and incubation in serum free media fo a further 6 hours or 22 hours (labelled 8 and 24 hours respectively to indicated total time from intial stimualtion). Total RNA was extracted and analysed for EPAC1 mRNA by RT-qPCR (A) and phase contrast microscopy (B). * and #: $p<0.05$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=3.

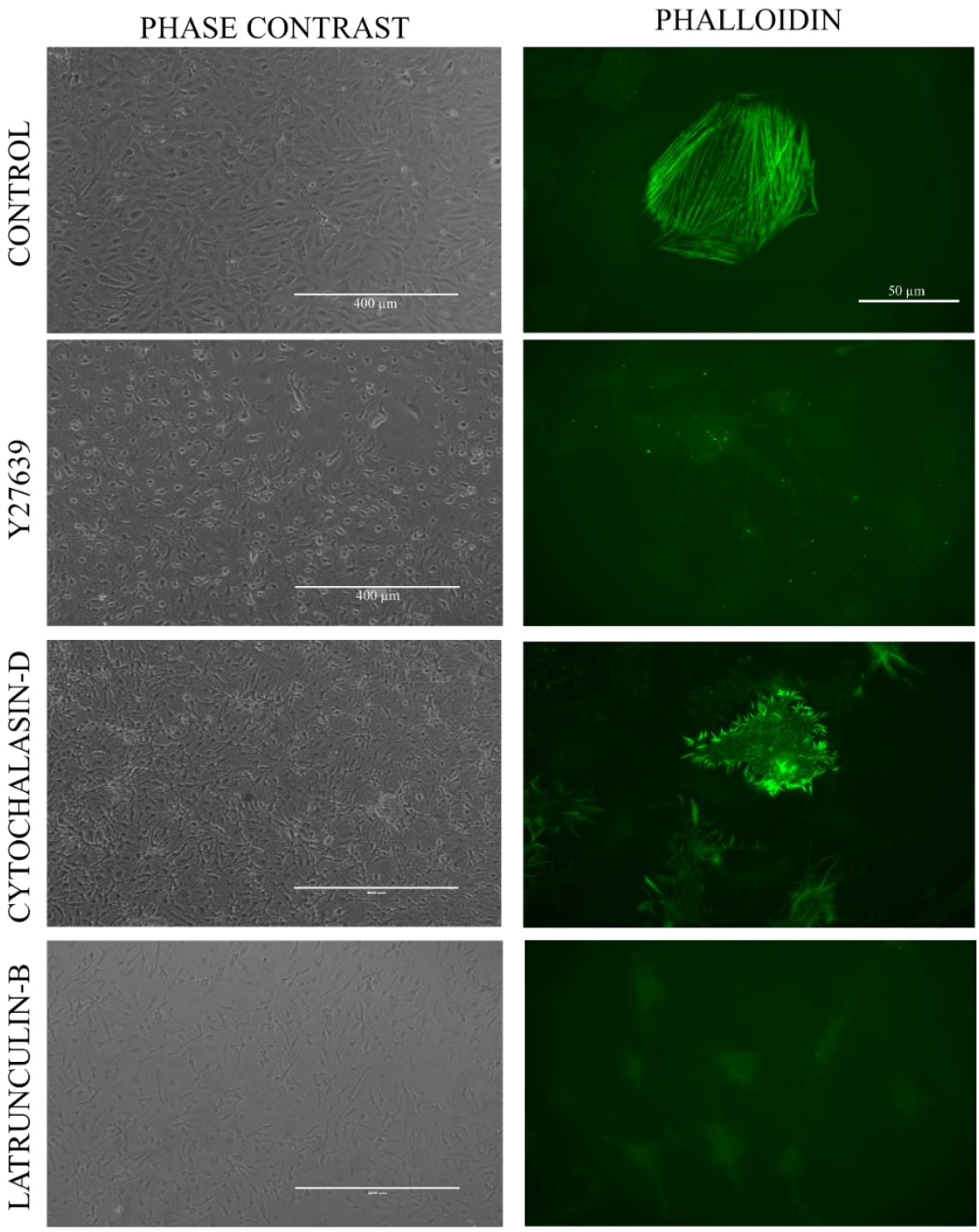


Figure 5.13: Actin cytoskeleton disruption induces stellate morphology and depolymerises actin fibres in cardiac fibroblasts

Serum-starved rat cardiac fibroblasts were stimulated with 10 µM Y27632, 2 µM cytochalasin-D (Cyto-D) or 0.5 ug/mL latrunculin-B (Lat-B) for 8 hours. Cell morphology and F-actin stress fibres were analysed by phase contrast microscopy and phalloidin staining, respectively.

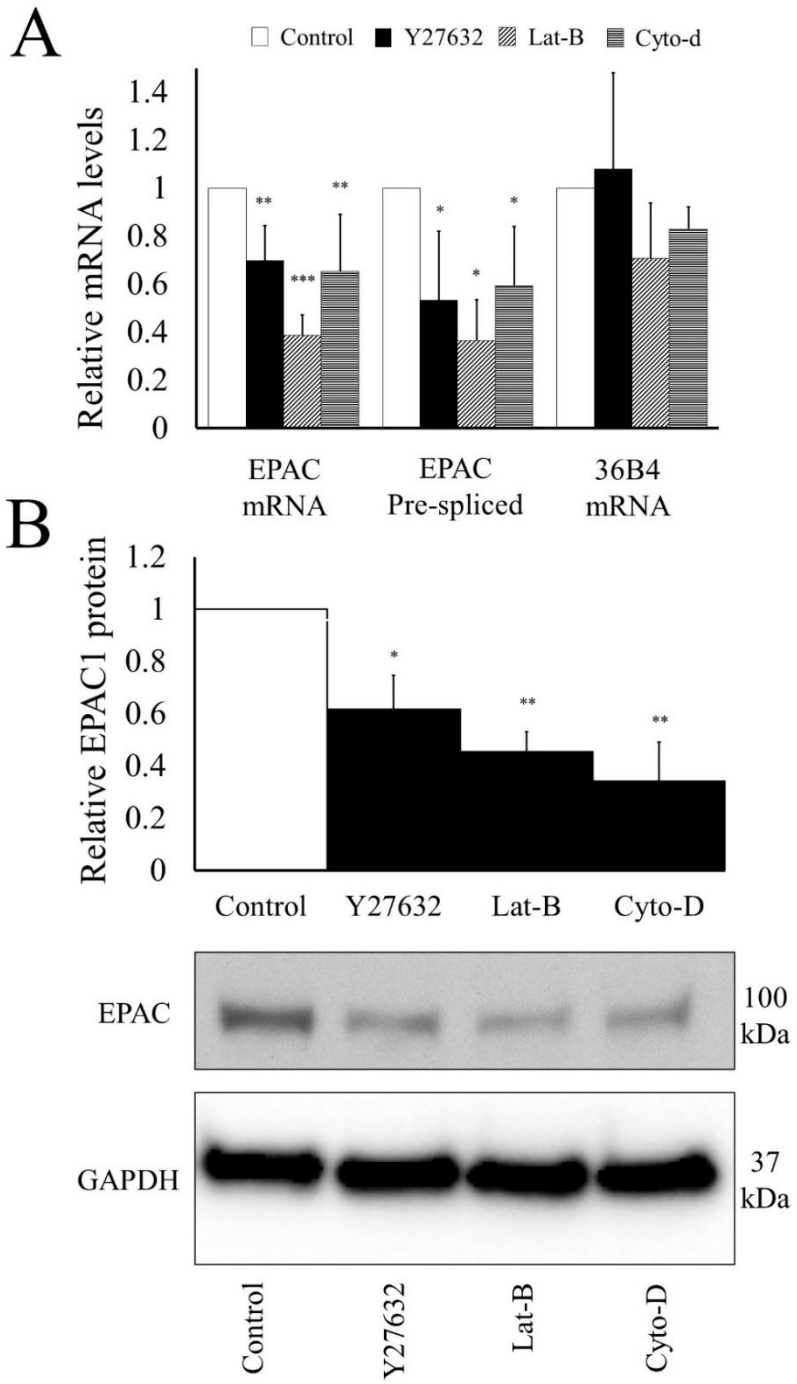


Figure 5.14: Actin cytoskeleton disruption inhibits EPAC1 expression.

Serum-starved rat cardiac fibroblasts were stimulated with 10 µM Y27632, 2 µM cytochalasin-D (Cyto-D) or 0.5 ug/mL latrunculin-B (Lat-B) for 8 hours Total RNA was extracted and analysed for EPAC1 mRNA, pre-spliced EPAC1 mRNA and 36B4 mRNA by qRT-PCR (C; n=3). Total cell lysates were analysed for EPAC1 and GAPDH protein levels by Western blotting and densitometric analysis (D; n=5). *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

5.3.3 cAMP-induced down regulation of EPAC1 impairs EPAC downstream signalling and cardiac fibroblasts morphological changes induced by cAMP signalling

Our next question was if cAMP-induced suppression of EPAC1 expression would result in impaired EPAC1 signalling and altered cellular responses induced by cAMP. To answer this question, cardiac fibroblasts were pre-stimulated with 25 µM forskolin for 24 hours to down regulate EPAC1 expression, which was confirmed by Western blotting (Figure 5.15A). Cardiac fibroblasts were then acutely stimulated with either 20 µM 8-CPT-cAMP-AM or 25 µM forskolin for 30 minutes and EPAC signalling quantified using RAP1 activity assays. In non-pre-treated cardiac fibroblasts, acute stimulation with either 8-CPT-cAMP-AM or forskolin resulted in an increase in $Rap1_{GTP}$ (active) levels (Figures 5.15C and 5.15D). However, in cardiac fibroblasts that were pre-treated with forskolin (for 24 hours), acute stimulation with 8-CPT-cAMP-AM or forskolin did not result in an increase in $Rap1_{GTP}$ (Figures 5.15C and 5.15D). Moreover, the morphological changes of cardiac fibroblasts induced by either forskolin or a combination of 8-CPT-cAMP-AM with 6-BNZ-cAMP-AM were significantly reduced in cells pre-stimulated with forskolin (Figures 5.16 and 5.17). These findings imply that reduction of EPAC1 expression caused by elevating cAMP stimuli, impairs EPAC1 down-stream signalling and morphological changes in response to subsequent cAMP signalling.

5.3.4 cAMP-induced inhibition of YAP-TEAD decreases EPAC1 promoter activity

Cardiac fibroblasts were transfected with a secreted nano-luciferase reporter gene driven by the human EPAC1 proximal promoter region (EPAC1-NLUC) to investigate if elevated levels of cAMP and actin-cytoskeleton disruption regulates EPAC1 levels at the level of transcription. Stimulation of EPAC1-NLUC transfected cardiac fibroblasts with 25 µM forskolin resulted in a significant inhibition of promoter activity without having effects on the control reporter gene driven by a minimal TNT-minimal promoter region (Figure 5.18A).

Furthermore, disruption of actin-cytoskeleton polymerisation using Y27631, Lat-B or Cyto-D also significantly inhibited EPAC1 promoter activity (Figure 5.18B). In order to detect potential transcription factors that might mediation this regulation, the human EPAC1 promoter was truncated into smaller fragments of 800 base pairs, 600 base pairs, 400 base pairs and 200 base pairs in length. Cardiac fibroblasts were transfected with these smaller human EPAC1 promoter truncations and stimulated with 25 µM forskolin. This resulted in a significant downregulation of the activities of all these smaller fragments (Figure 5.18C), suggesting the presence of a cAMP sensitive transcription factor within the proximal 200 bp of the human EPAC1 promoter.

Sequence analysis of this proximal 200 base pair region identified a conserved binding element for the transcription factor (TEAD). A large body of research has previously documented the association of actin-cytoskeleton organisation to regulation of TEAD activity (Dupont, Morsut et al. 2011, Reddy, Deguchi et al. 2013, Zhang, Lin et al. 2014, Kimura, Duggirala et al. 2016). As a result of this, we tested if cAMP-dependent regulation of EPAC1 expression in cardiac fibroblasts was regulated by TEAD activity.

At first, we asked if elevation of cAMP levels or disruption of actin cytoskeleton polymerisation would result in downregulation of TEAD activity and expression of TEAD target gene mRNAs. In order to test this, cardiac fibroblasts were transfected with a synthetic TEAD reporter gene and stimulated with cAMP elevating agents. Stimulation of these cells with BAY60-6583, forskolin or Db-cAMP analogue all resulted in a significant inhibition of TEAD-dependent reporter gene activity. Importantly, the activity of the TEAD independent minimal TNT-promoter (control promoter) was not affected (Figure 5.19A). We also tested if direct disruption of actin-cytoskeleton polymerisation with latrunculin-B, cytochalasin-D or Y27632 also resulted in a reduction in TEAD luciferase reporter gene activity. Stimulation of TEAD-LUC transfected cells with Lat-B, Cyto-D or Y27632 all significantly inhibited TEAD reporter activity (Figure 5.19B). Finally, we tested in elevated cAMP also inhibited mRNA expression of the classical TEAD-target genes, CCN1 and CTGF (Kimura, Duggirala et al. 2016, Smith, Sessions et al. 2019). Forskolin stimulation for 2 or 8 hours resulted in a significant downregulation of both CCN1 and CTGF mRNA and pre-spliced RNA levels, indicating transcriptional repression of these genes by forskolin (Figure 5.19C).

As mentioned in chapter 1, the activity of TEAD is regulated by its transcriptional co-factor YAP (Vassilev, Kaneko et al. 2001, Zhao, Li et al. 2010). Immunofluorescent staining of cardiac fibroblasts stimulated with 25 µM forskolin, identified a significant reduction in nuclear and cytoplasmic levels of YAP protein (Ebrahimighaei, McNeill et al. 2019). In addition, western blot analysis of total cardiac fibroblast lysates further demonstrated a significant and rapid downregulation of total YAP protein levels (Figure 4.18), and a significant upregulation in the level of YAP phosphorylation at serine 172 and serine 397 (Figure 4.18). Previously, it was documented that these modifications are associated with export of YAP from nucleus and its degradation (Zhao, Wei et al. 2007, Zhao, Li et al. 2010). Taken everything into consideration, these results show that EPAC1 and TEAD promoter activities are inhibited by cAMP elevating stimuli and actin cytoskeleton disruption.

We next asked if the TEAD element present in the EPAC1 proximal promoter is functionally important for the EPAC1 expression in cardiac fibroblasts. We initially tested if this TEAD element could bind TEAD protein using electromobility shift assay (EMSA) analysis. EMSA analysis of the EPAC1 TEAD element showed that nuclear extracts from Myc-TEAD1 transfected cardiac fibroblasts, but not control transfected cells, formed a band of reduced electrophoretic mobility when incubated with a biotinylated DNA probe containing the wild-type EPAC1 promoter TEAD element, suggesting that TEAD1 is bound to the TEAD element in the human EPAC1 promoter (Figure 5.20A). In contrast, this band of reduced mobility was formed when a 3 base-pair mutation was introduced into the TEAD element. This implies that, TEAD1 protein binds specifically to the consensus TEAD sequence in the human EPAC1 promoter (Figure 5.20A).

In the context of a luciferase reporter gene, the introduction of these 3 base pair mutation in the consensus TEAD element significantly inhibited EPAC1 promoter activity (Figure 5.20B), indicating that this TEAD element is functionally important for maximal EPAC1 promoter activity in cardiac fibroblasts.

In order to further test the hypothesis that the YAP-TEAD transcriptional complex is important for EPAC1 expression, we used siRNA-mediated gene silencing to silence the TEAD co-factors YAP and TAZ. Silencing these co-transcription factors resulted in a strong down regulation of YAP and TAZ protein expression without affecting levels of GAPDH or β-actin protein (Figure 5.21A). A similar silencing of YAP and TAZ mRNA, but not 36B4 mRNA, was detected after transfection with YAP and TAZ siRNA, indicating efficient and specific gene silencing (Figure 5.21B). Importantly, silencing of YAP and TAZ resulted in a significant downregulation of the mRNA levels of the classical TEAD-target genes CCN1 and CTGF as well as significantly reducing EPAC1 mRNA and EPAC pre-spliced RNA levels without affecting the mRNA levels of the housekeeping gene, 36B4 (Figure 5.21B). In the context of the reporter gene assay, silencing YAP and TAZ significantly reduced the TEAD-reporter gene and EPAC1 promoter activity (Figures 5.21C and 5.21D). These findings highlight the roles of YAP/TAZ-TEAD in regulating the expression of EPAC1.

In order to investigate the regulatory role of TEAD on EPAC1 expression further, we transfected cardiac fibroblasts with a dominant-negative TEAD1 expression vector that expresses the TEAD1 DNA binding domain fused to the *Drosophila* engrailed transcriptional repressor domain (TEAD1-Eng). Expression of TEAD1-Eng significantly reduced TEAD reporter gene activity without inducing significant changes on the activity of the control promoter, which lacks any TEAD-binding

elements (Figure 5.22A), consistent with specific inhibition of TEAD activity. Importantly, expression of TEAD1-Eng also significantly inhibited EPAC1 reporter gene activity, without affecting activity of a minimal promoter reporter gene (Figure 5.22B).

The formation of a YAP-TEAD complex is a crucial step in the transcription of TEAD-dependent genes. Therefore, in order to further test the roles of TEAD in the regulation of EPAC1 gene expression, we employed two different pharmacological inhibitors of YAP/TAZ-TEAD complex, namely Verteporfin (Liu-Chittenden, Huang et al. 2012) and compound 3.1 (CPD3.1) (Smith, Sessions et al. 2019). Stimulation of cardiac fibroblasts with Verteporfin or CPD3.1 significantly reduced TEAD-NLUC activity (Figure 5.23A). Likewise, treatment of cardiac fibroblasts with the two TEAD inhibitors significantly inhibited EPAC1 promoter activity (Figure 5.23B). In addition, cardiac fibroblast stimulation with Verteporfin and CPD3.1, induced a downregulation on the mRNA levels of the TEAD-target genes, CCN1 and CTGF, as well as retarding the levels of EPAC1 mRNA and its pre-spliced form (Figures 5.24A and 5.24B). In contrast, levels of the TEAD-independent housekeeping genes 36B4 or TBP were not affected by either Verteporfin or CPD3.1 stimulation (Figures 5.24A and 5.24B). Taken together these data demonstrated that YAP/TAZ-TEAD activity is required for maximal EPAC1 gene expression in cardiac fibroblasts.

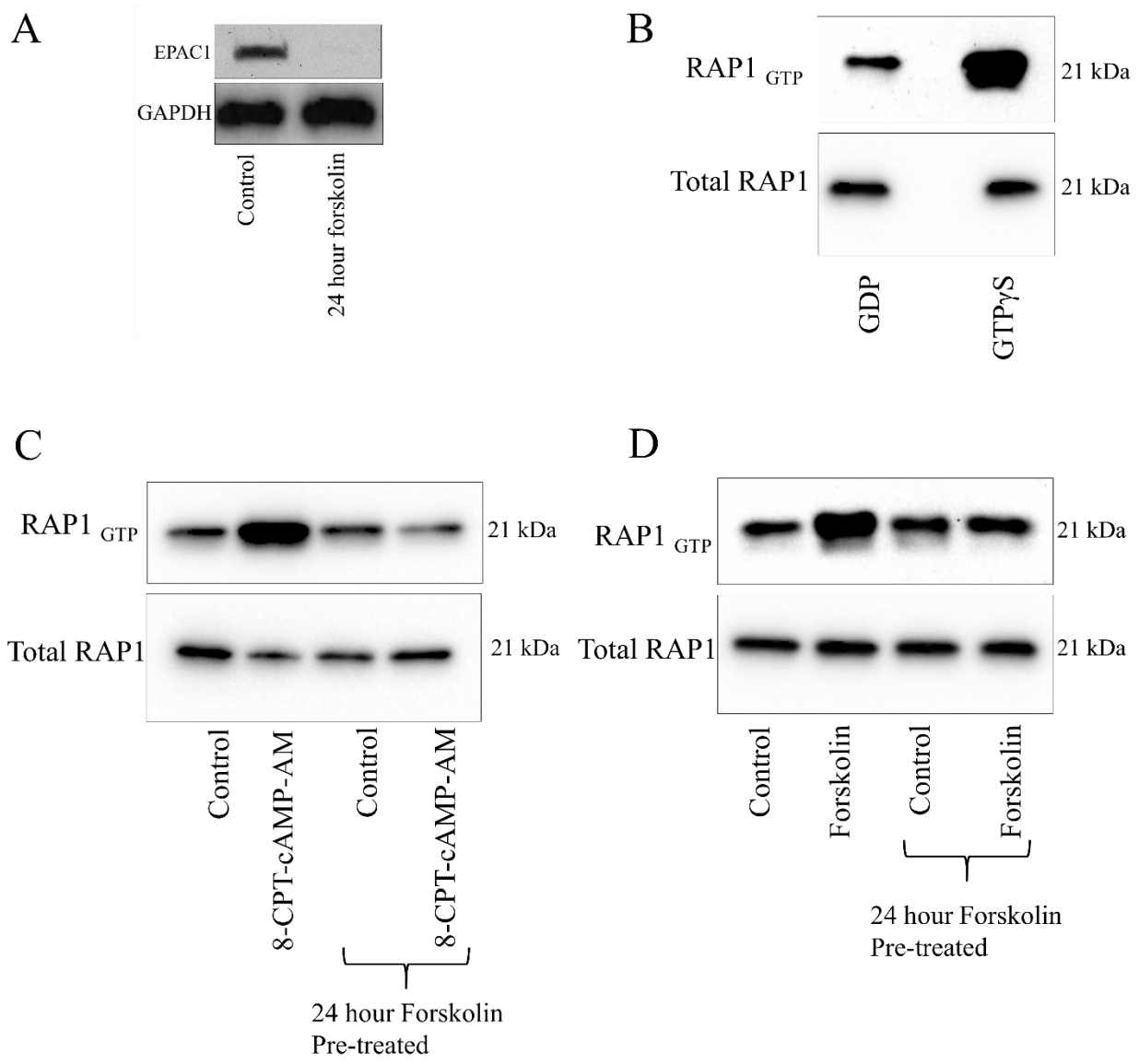


Figure 5.15: Chronic forskolin stimulation attenuates induction of RAP1$_{GTP}$ levels in response to subsequent cAMP-elevating stimuli.

Serum-starved rat cardiac fibroblasts were stimulated with 25 µM forskolin for 24 hours, as indicated. Total cell lysates were analysed for EPAC1 and GAPDH protein levels (**A**). Rap1 assay GDP (negative) and GTPγS (positive) controls (**B**). Forskolin pre-treated or non-pre-treated cardiac fibroblast were stimulated with 20 µM 8-pCPT-2'-O-Me-cAMP-AM (**C**) or 25 µM forskolin (**D**) for 30 minutes. RAP1$_{GTP}$ (active) and total RAP1 levels were detected by RAP1 pull-down assays and Western blotting (**C-D**), n=1.

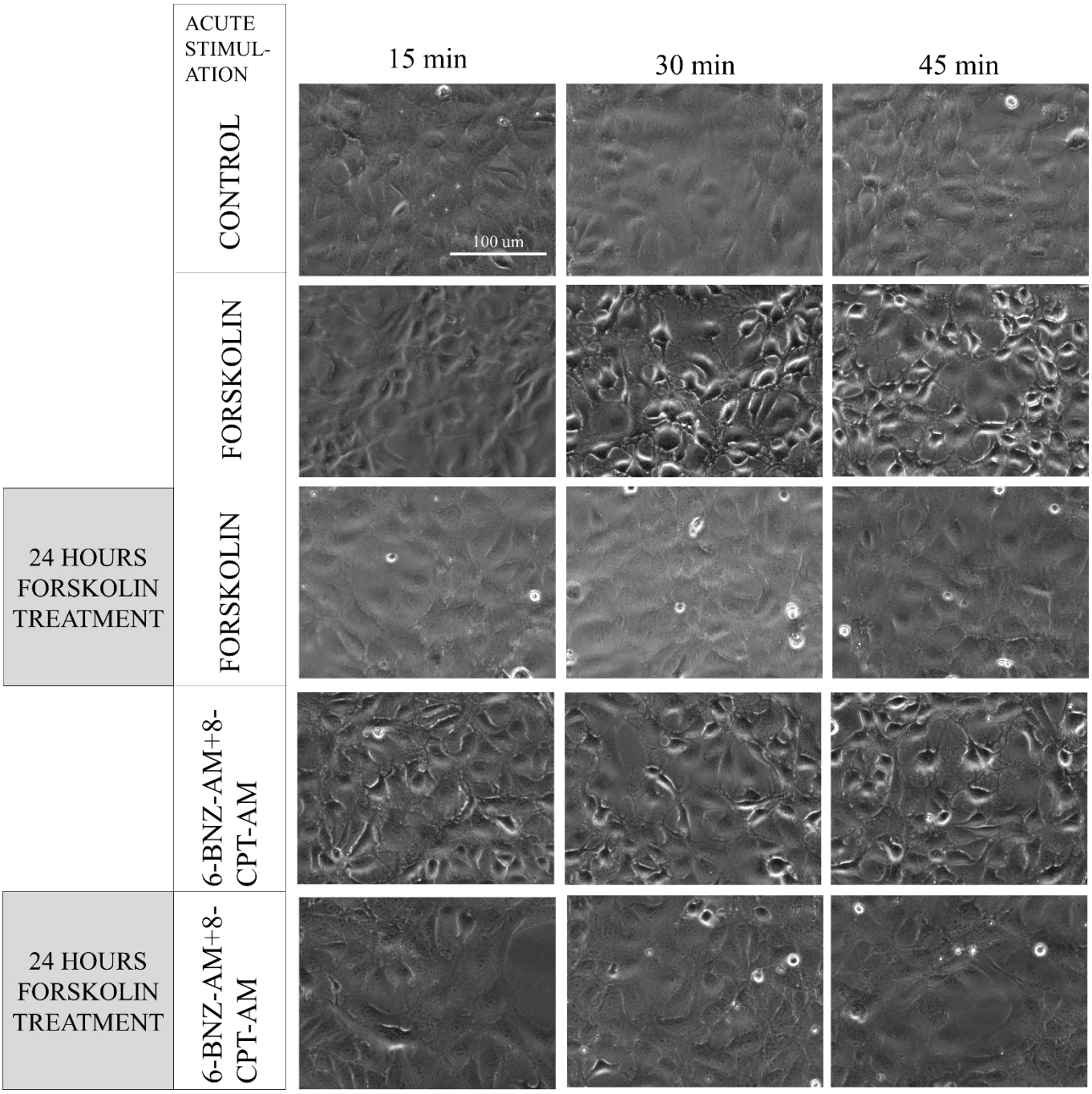


Figure 5.16: Chronic forskolin stimulation attenuates induction of morphological change in response to subsequent cAMP-elevating stimuli.

Serum-starved rat cardiac fibroblasts were pre-treated with either serum free media or 25 µM forskolin for 24 hours, as indicated. Cells were then stimulated for 15, 30 or 45 minutes with 25 µM forskolin or 20 µM 8-pCPT-2'-O-Me-cAMP-AM plus 20 µM 6-BNZ-cAMP-AM. Cell morphology was analysed by phase contrast microscopy. Scale bar indicate 100 µm.

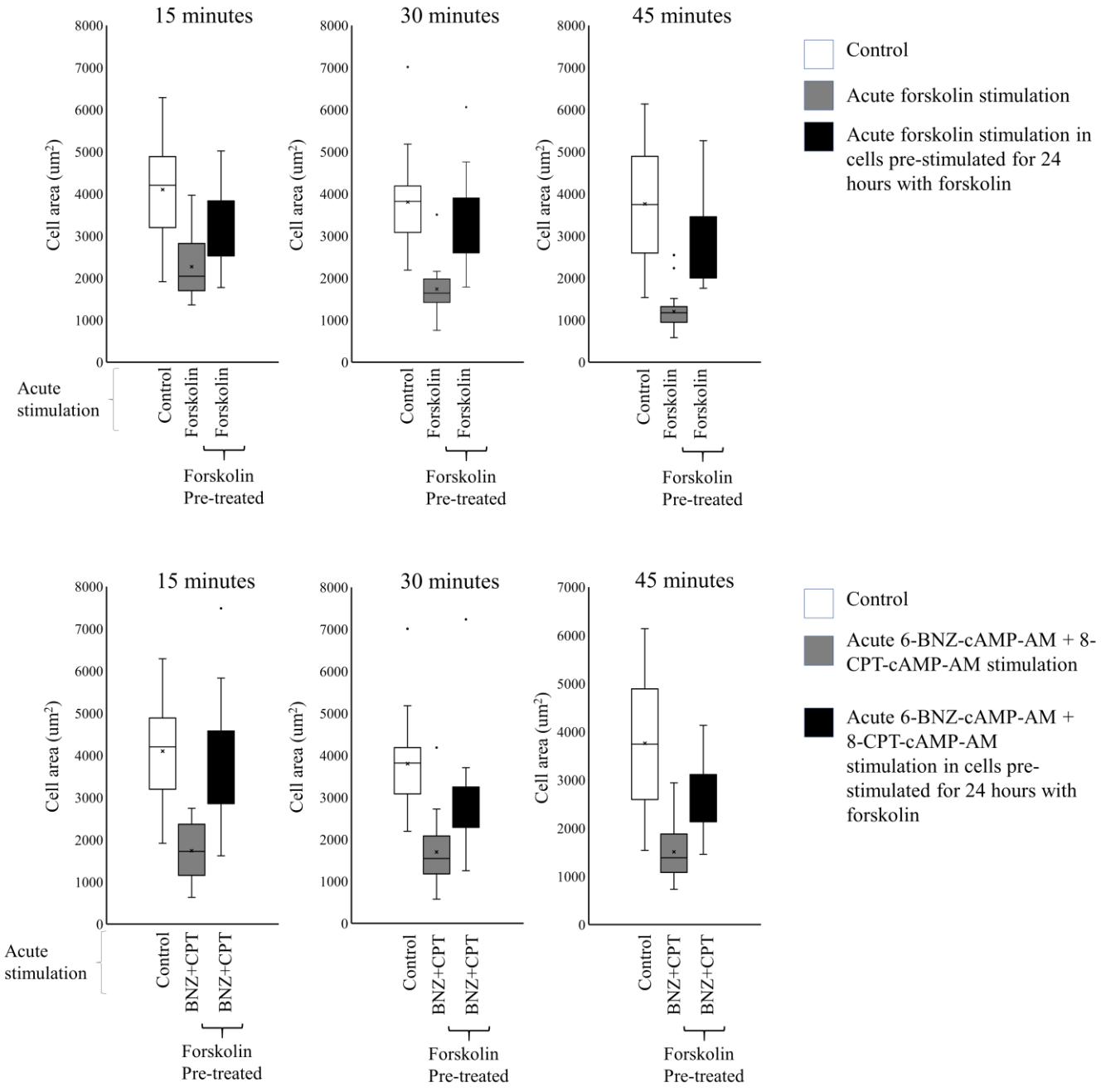


Figure 5.17: Chronic forskolin stimulation attenuates induction of morphological change in response to subsequent cAMP-elevating stimuli.

Serum-starved cardiac fibroblasts were pre-treated with either serum free media or 25 µM forskolin for 24 hours, as indicated. Cells were then stimulated for 15, 30 or 45 minutes with 25 µM forskolin or 20 µM 8-pCPT-2'-O-Me-cAMP-AM (CPT) plus 20 µM 6-BNZ-cAMP-AM (BNZ). Cell area was quantified by image analysis of phase contrast micrographs. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

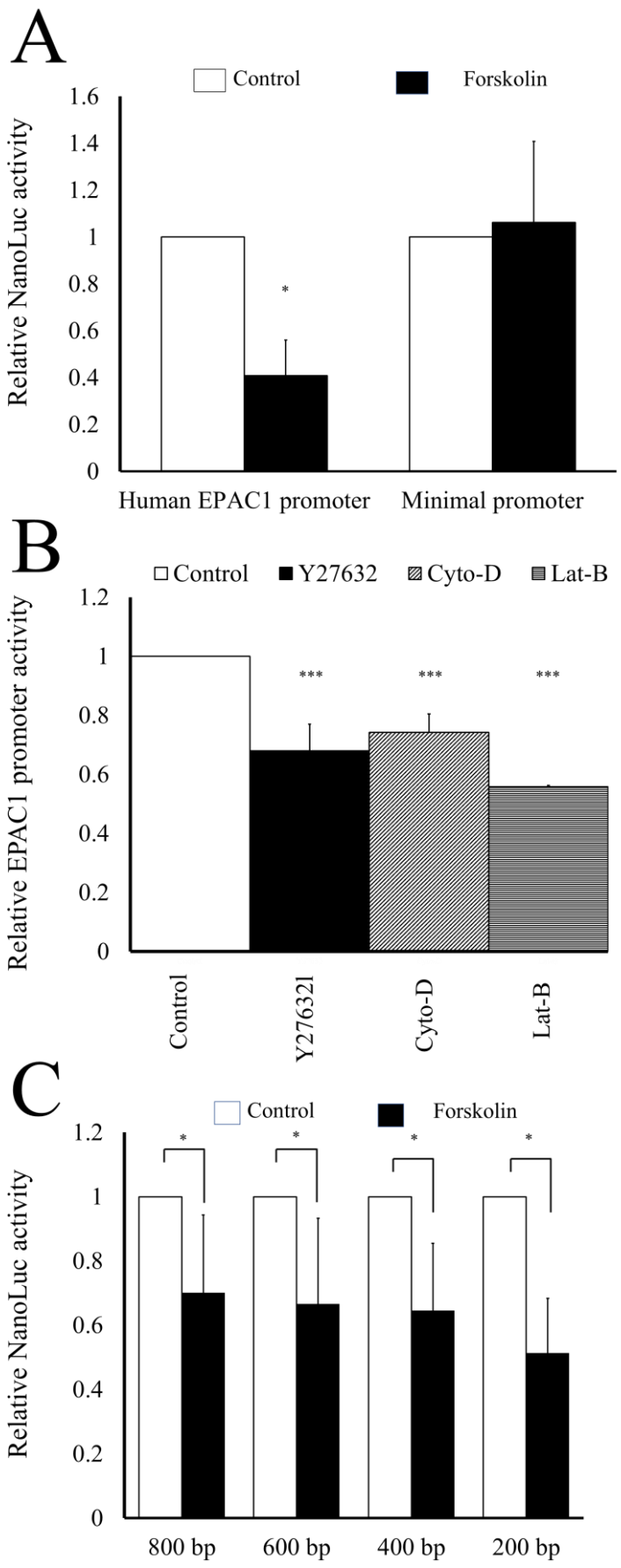
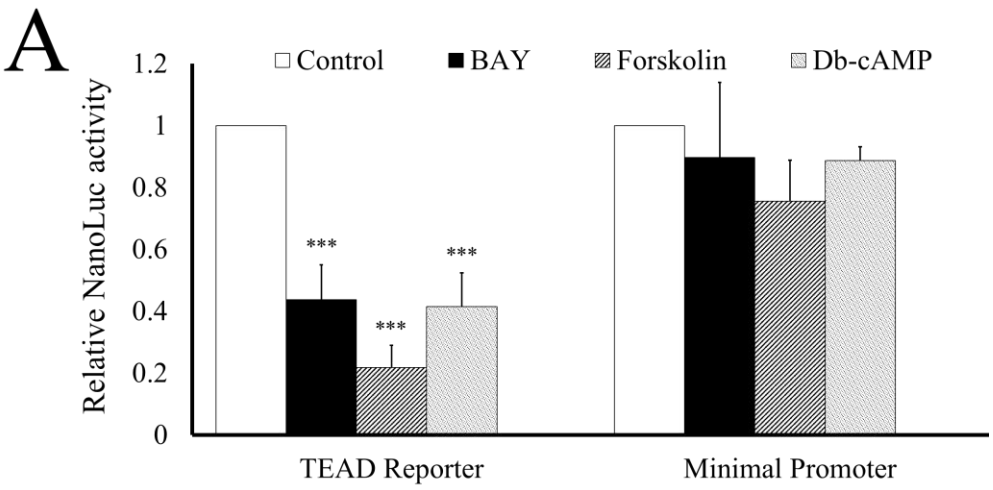
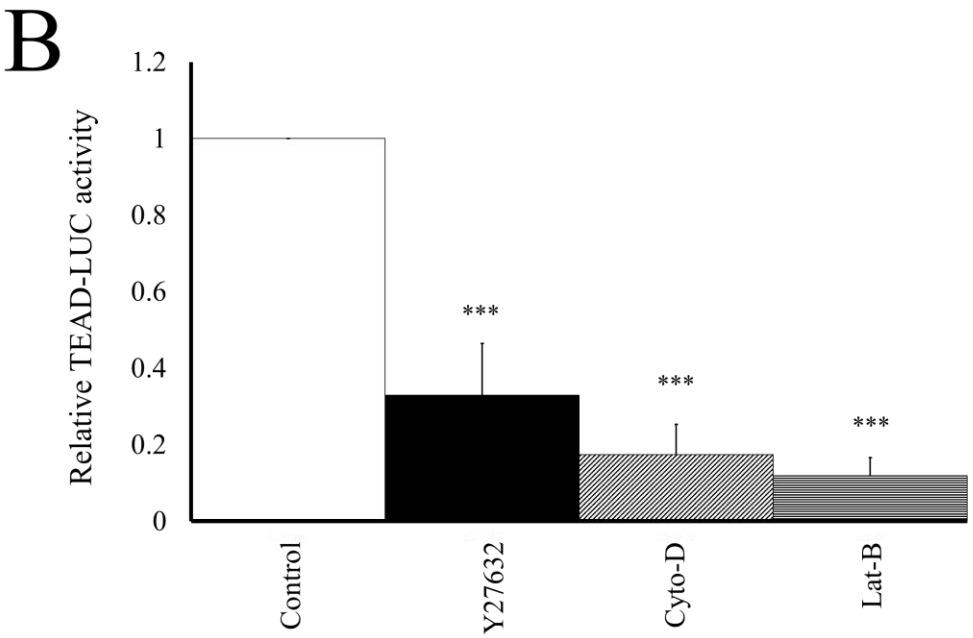

A
Control
Forskolin
Relative NanoLuc activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Human EPAC1 promoter
Minimal promoter
B
Control
Y27632
Cyto-D
Lat-B
Relative EPAC1 promoter activity
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
Control
Y27632l
Cyto-D
Lat-B
C
Control
Forskolin
Relative NanoLuc activity
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
800 bp
600 bp
400 bp
200 bp

Figure 5.18: Elevated cAMP and inhibition of actin polymerisation reduces EPAC1 promoter activity and TEAD activity in cardiac fibroblasts.

Rat cardiac fibroblasts were transfected with EPAC1-NLUC reporter (**A** and **B**) and stimulated with 25 µM forskolin, 10 µM Y27632, 2 µM Cyto-D or 0.5 µg/mL Lat-B for 8 hours. Cells were transfected with a NLUC reporter vector controlled by the indicated EPAC1 promoter truncations or a minimal promoter, as indicated, and NLUC activity assayed 24 hours later (**C**). **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.


A
Control
BAY
Forskolin
Db-cAMP
Relative NanoLuc activity
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
TEAD Reporter
Minimal Promoter


B
Relative TEAD-LUC activity
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
Control
Y27632
Cyto-D
Lat-B

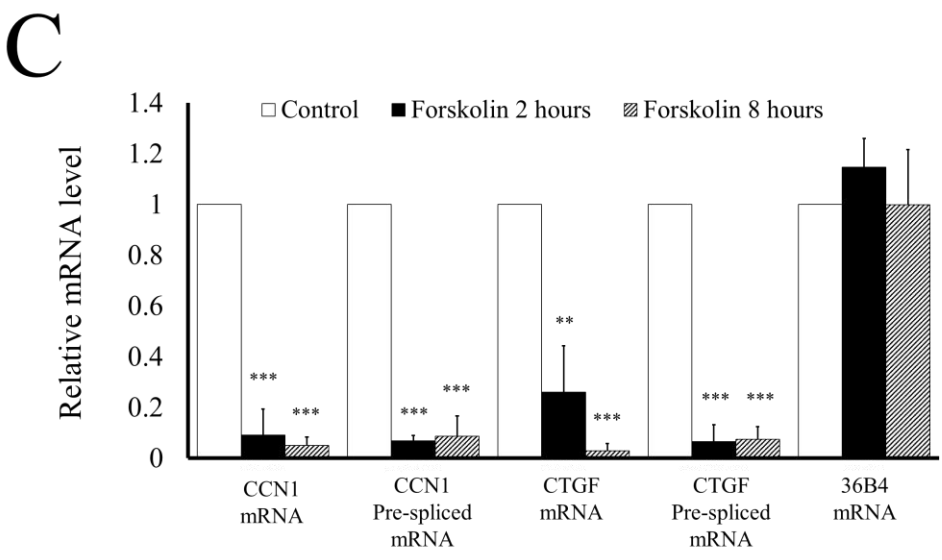

C
Control
Forskolin 2 hours
Forskolin 8 hours
Relative mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
***
**
***
***
***
CCN1 mRNA
CCN1 Pre-spliced mRNA
CTGF mRNA
CTGF Pre-spliced mRNA
36B4 mRNA

Figure 5.19: Elevated cAMP and inhibition of actin polymerisation TEAD activity in cardiac fibroblasts.

Rat cardiac fibroblasts were transfected with a TEAD-LUC reporter or minimal promoter-LUC reporter vector as indicated and stimulated with 5 µg/mL BAY65-6085, 25 µM forskolin, 200 µM db-cAMP and luciferase activity quantified after 8 hours (**A**). Cardiac fibroblasts were transfected with a TEAD-LUC reporter vector and stimulated with 10 µM Y27632, 2 µM Cyto-D and 0.5 µg/ml Lat-B and luciferase activity was quantified after 8 hours (**B**). Cardiac fibroblasts were stimulated with 25 µM forskolin for 2 and 8 hours (**C**). Total RNA was extracted and analysed for CCN1 mRNA, pre-spliced CCN1 RNA, CTGF mRNA, pre-spliced CTGF RNA and 36B4 mRNA levels (F). **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

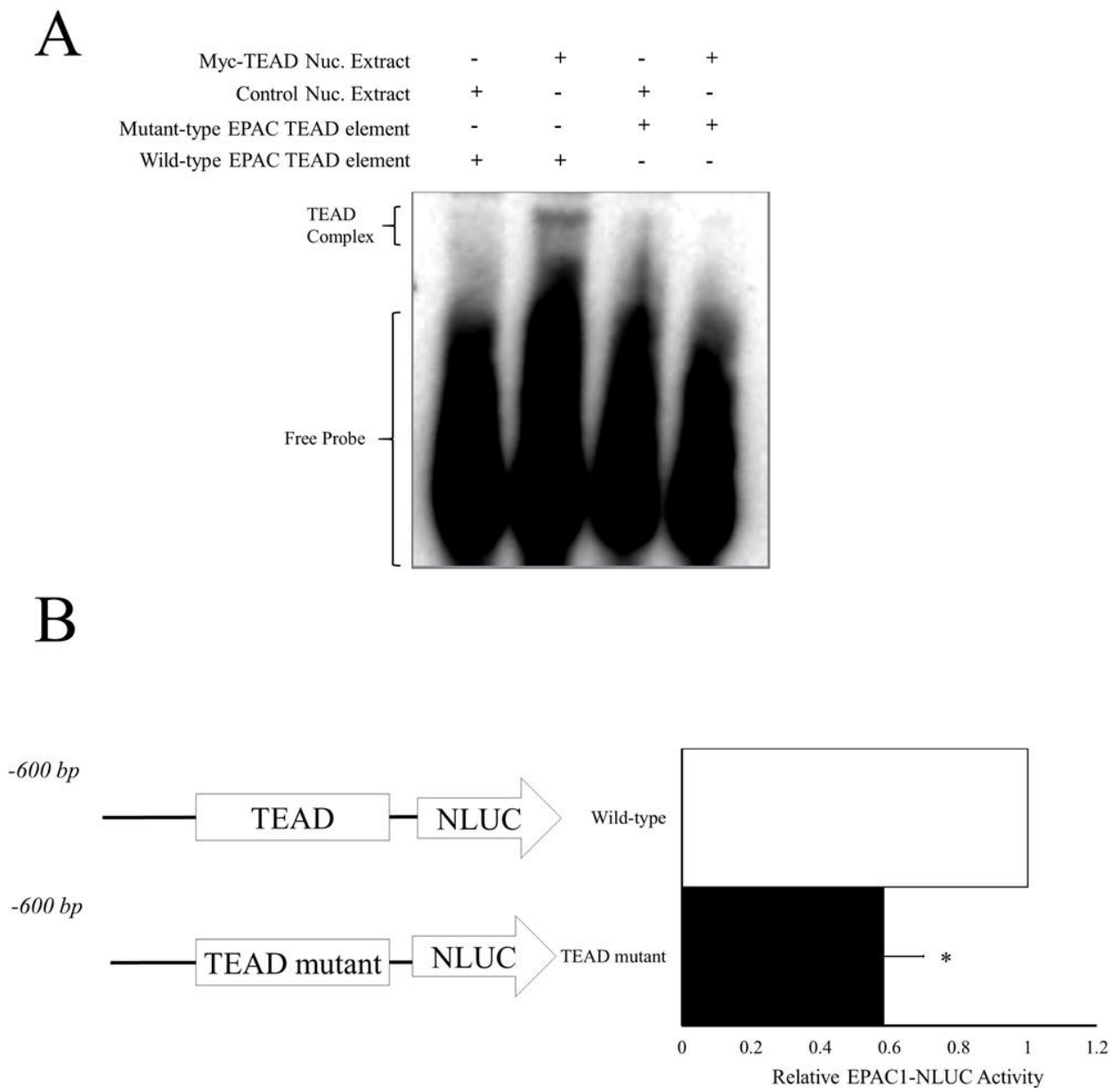


Figure 20: YAP/TAZ-TEAD activity is required for the maximal expression of EPAC1.

Electromobility shift assay (EMSA) analysis of binding of TEAD protein from nuclear extracts from control or myc-TEAD1 transfected rat cardiac fibroblasts to DNA probes containing either the wild-type or mutated TEAD element from the EPAC1 proximal promoter (**A**). Rat cardiac fibroblasts were transfected with a secreted nanoluciferase reporter (NLUC) gene plasmid under the control of either the wild-type EPAC1 promoter or the EPAC1 promoter containing a mutated TEAD element. Secreted NLUC activity was quantified 24 hours after transient transfection (**B**). *: $p<0.05$. Data are expressed as mean ±SEM and analysed by paired Students t-test, n=4.

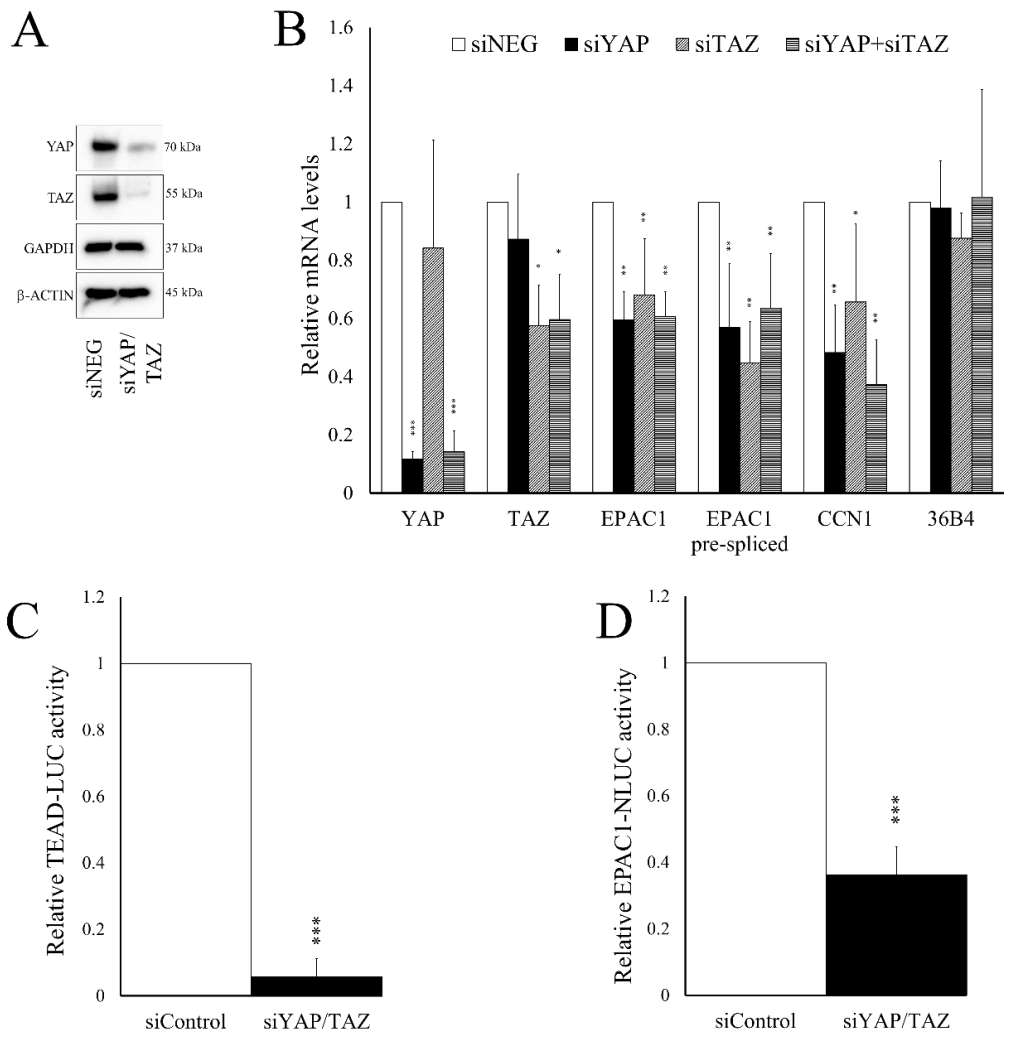


Figure 5.21: YAP/TAZ-TEAD activity is required for maximal EPAC1 expression

Rat cardiac fibroblasts were transfected with siRNA targeting YAP and TAZ (siYAP/TAZ) or non-targeting control siRNA (siControl), together with TEAD-LUC or EPAC1-NLUC reporter plasmids. Total cell lysates were analysed for protein levels of YAP, TAZ, GAPDH or β-ACTIN by Western blotting (**A**). Total RNA was extracted and analysed for the mRNA levels of YAP, TAZ, EPAC1, pre-spliced EPAC1 mRNA, CCN1 and 36B4 using RT-qPCR (**B**). Total cell lysates were analysed, and reporter gene activity quantified (**C** and **D**). *: $p<0.05$; **: $p<0.01$ and ***: $p<0.01$. Data are expressed as mean ±SEM and analysed by paired Students t-test (**C** and **D**) or ANOVA with Student Newman Keul's post-test (**B**), n=4.

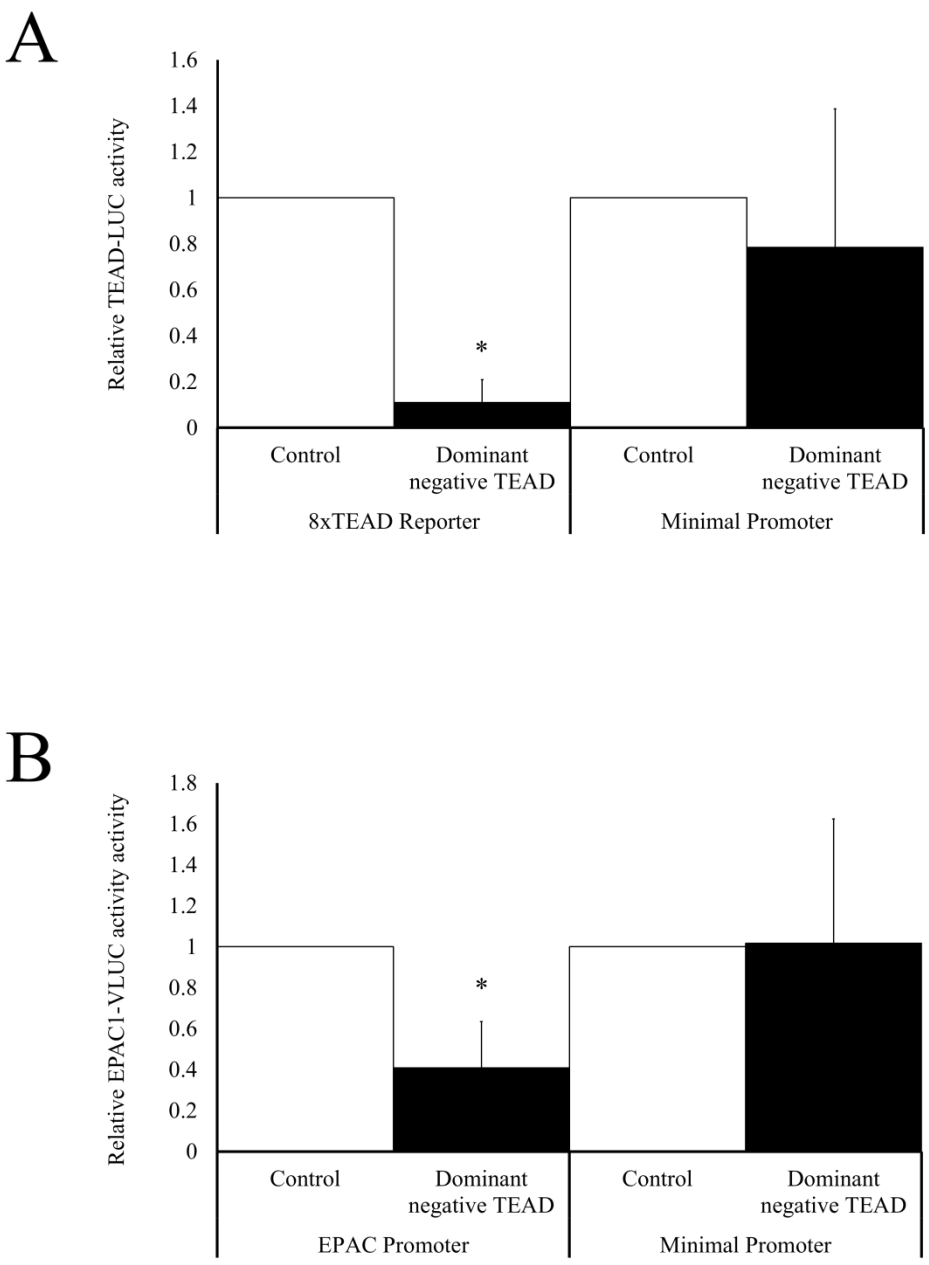


Figure 5.22. YAP/TAZ-TEAD activity is required for maximum EPAC1 activity.

Rat cardiac fibroblasts were transfected with TEAD-LUC (**A**), EPAC1-NLUC (**B**) or a minimal promoter reporter (**A** and **B**), together with either an empty expression vector lacking a transgene (Control) or a dominant-negative TEAD expression vector, as indicated (**A** and **B**). Reporter gene activity was quantified 24 hours after transfection. *: $p<0.05$. Data are expressed as mean ±SEM and analysed by paired Students t-test, n=4

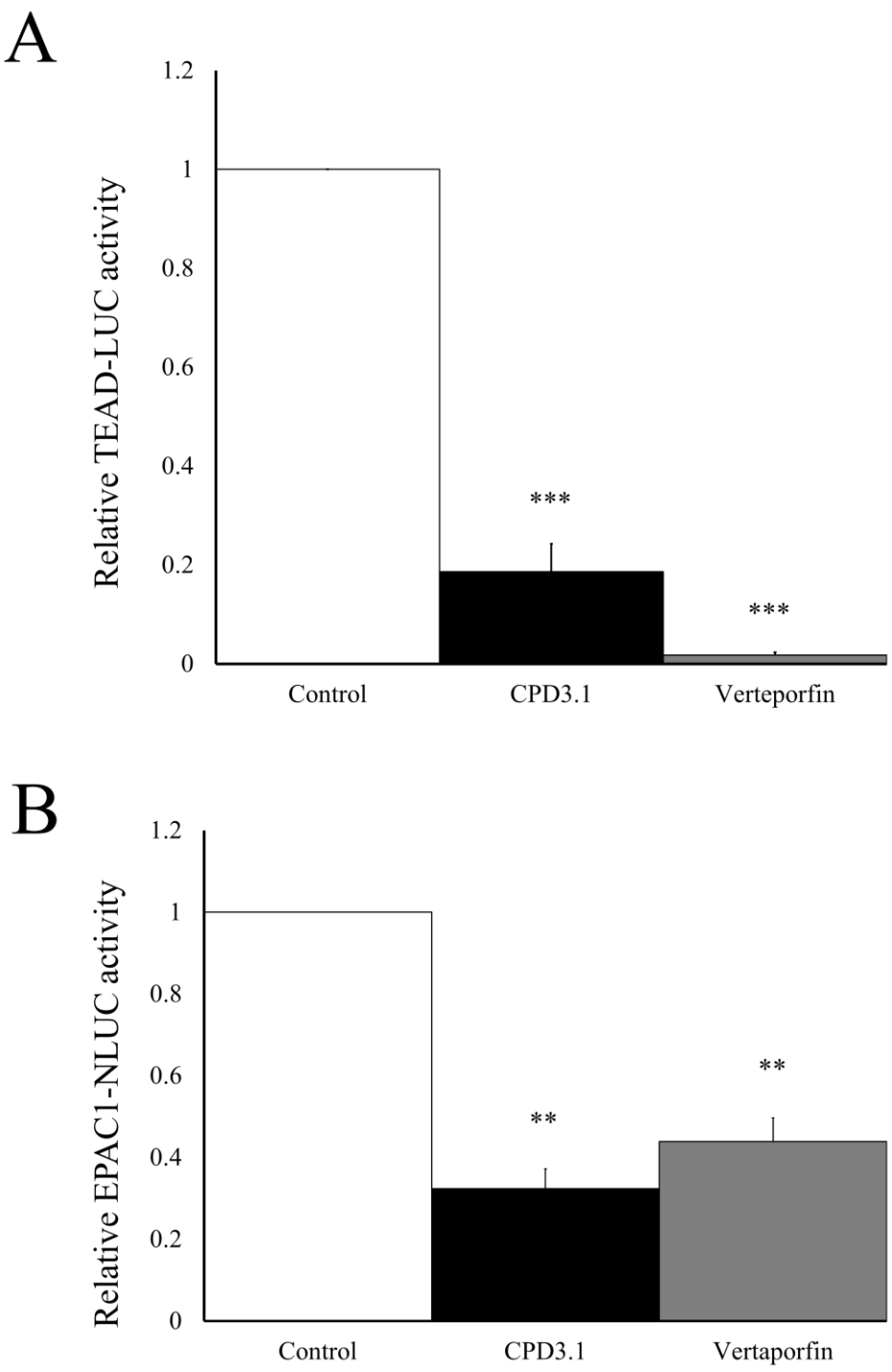


Figure 5.23: Pharmacological TEAD inhibition reduces human EPAC1 promoter.

Rat cardiac fibroblasts were transfected with TEAD-LUC reporter (**A**) or EPAC1-NLUC reporter (**B**) and stimulated with either 60 µM CPD 3.1 or 10 µM verteporfin. Reporter gene activity was quantified 24 hours after transfection. **: $p<0.01$ and ***: $p<0.01$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

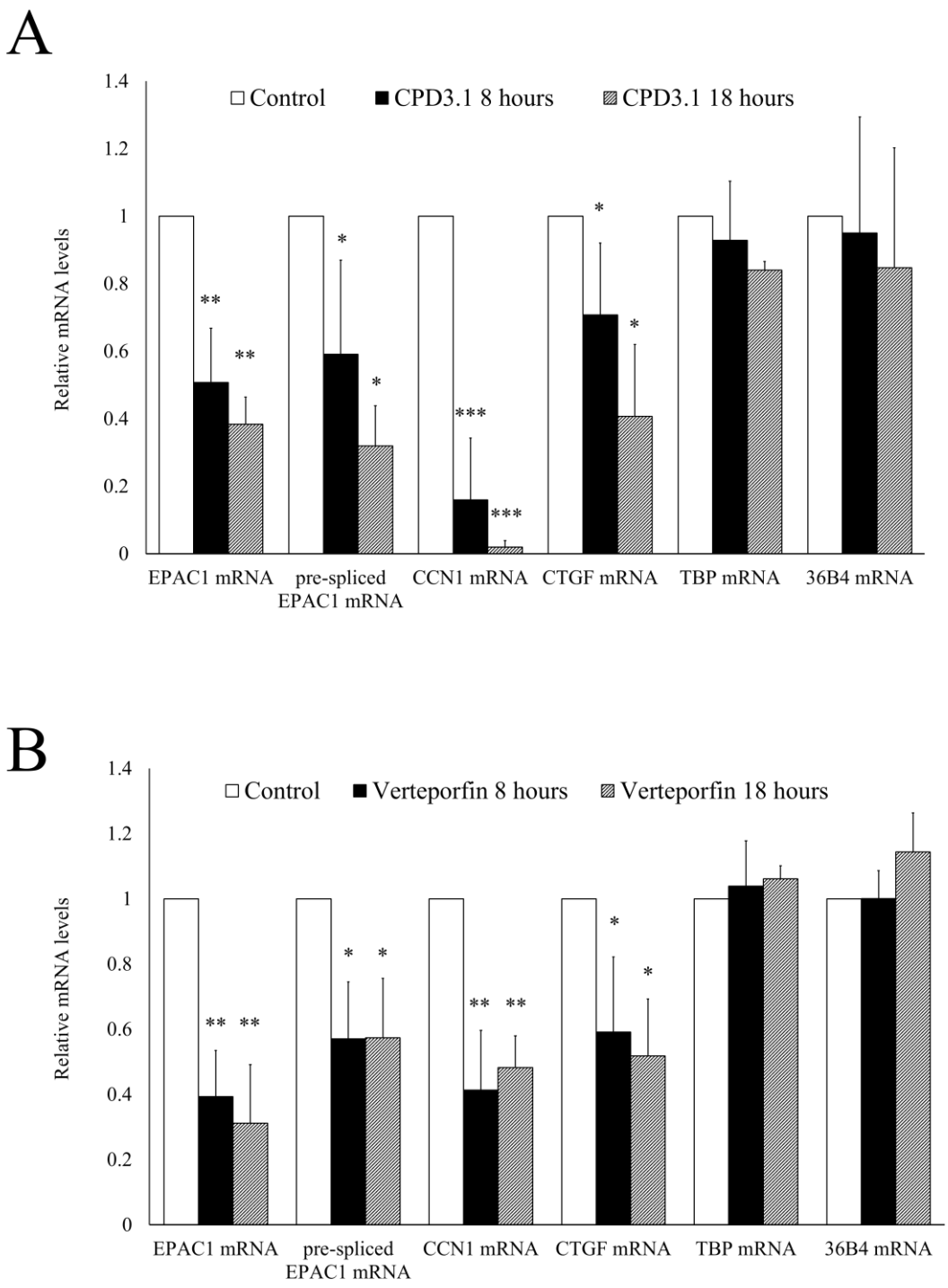


Figure 5.24: Pharmacological TEAD inhibition reduces EPAC1 and TEAD target genes.

Rat cardiac fibroblasts were stimulated with 60 µM CPD3.1 for 8 or 18 hours (**A**) or 10 µM Verteporfin (**B**) and total RNA was extracted and analysed for the mRNA levels of EPAC1, pre-spliced EPAC1, CCN1, CTGF, TBP and 36B4 mRNA by RT-qPCR. *: $p<0.05$; **: $p<0.01$ and ***: $p<0.01$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

5.3.5 YAP and TAZ are required but insufficient for EPAC1 mRNA expression

To further test the importance of YAP and TAZ in the regulation of EPAC1 expression, we asked if YAP and TAZ activation is sufficient to reverse the inhibitory effects of elevated cAMP levels on EPAC1 expression. To test this, we over expressed the constitutively active mutants of YAP (YAP_{S127A}) and TAZ (TAZ_{5SA}) using adenoviruses. $YAPS_{127A}$ over expression completely rescued forskolin-mediated inhibition of TEAD-reporter activity, confirming efficient reversal of cAMP mediated inhibition of TEAD reporter activity (Figure 5.25A). Likewise, over expression of $YAPS_{127A}$ completely reversed the cAMP-mediated inhibition of EPAC1 promoter activity (Figure 5.25B), which is consistent with our findings showing the requirement for YAP-TEAD activity for maximal EPAC1 expression.

We next tested if YAP over expression could also reverse mRNA levels of TEAD target genes (CCN1) and EPAC1 following forskolin stimulation. Forskolin treatment of cardiac fibroblasts infected with a control adenovirus lacking a transgene, induced a reduction in the mRNA levels of CCN1 (Figure 5.26A). In addition, this downregulation was completely rescued by the over expression of either YAP_{S127A} or TAZ_{5SA}, consistent with our data on the rescue of TEAD-dependent reporter gene activity (Figure 5.26A). Similarly, forskolin stimulation of cardiac fibroblasts infected with control adenovirus resulted in downregulation of EPAC1 mRNA levels (Figure 5.26B). However, in contrast to CCN1 mRNA levels, over expression of YAP_{S127A} or TAZ_{5SA} co-transcription factors did not reverse the forskolin-induced inhibition effects on the mRNA levels of EPAC1 (Figure 5.26B).

Taken together, these data suggest that YAP or TAZ-mediated activation of TEAD alone is not sufficient for EPAC1 mRNA expression, despite the inhibitory effects of dominant negative TEAD and silencing of YAP and TAZ on the mRNA levels of EPAC1 in cardiac fibroblasts (Figures 5.21 and 5.22). This implies the contribution of at least one additional mechanism that is necessary for expression of the endogenous EPAC1 gene that is not essential for regulation of the EPAC1 reporter gene activity.

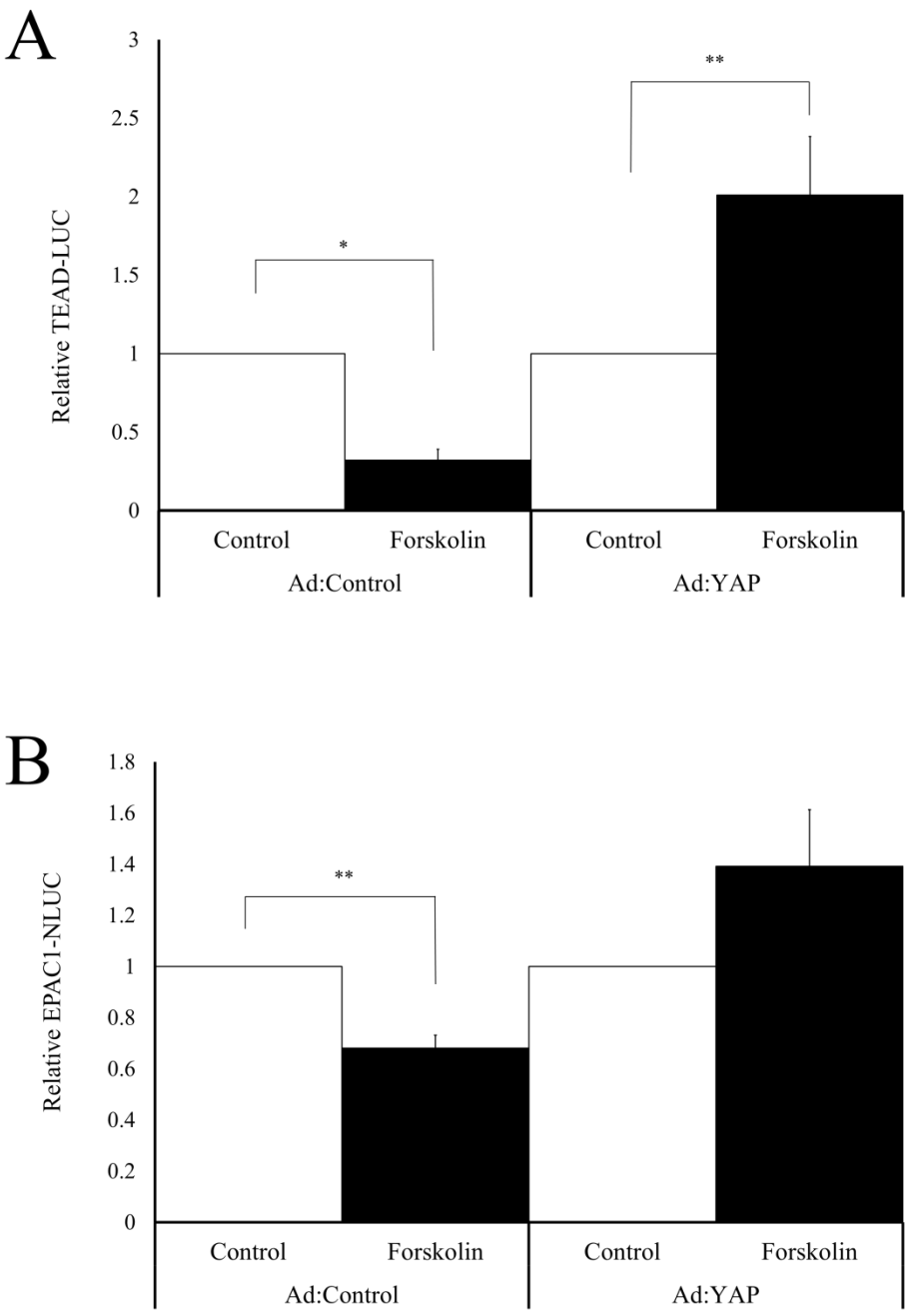


Figure 5.25. YAP over expression rescues forskolin-mediated inhibition of TEAD reporter gene and EPAC1 promoter activity.

Rat cardiac fibroblasts were transfected with TEAD-LUC (**A**) or EPAC-NLUC (**B**) reporter gene plasmids. The next day, cells were infected with either a control adenovirus (Ad:Control) or adenovirus expressing constitutively-active YAP. Cells were then stimulated with 25 µM forskolin for 8 hours and reporter gene activity quantified. *: $p<0.05$ and ** $p<0.01$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

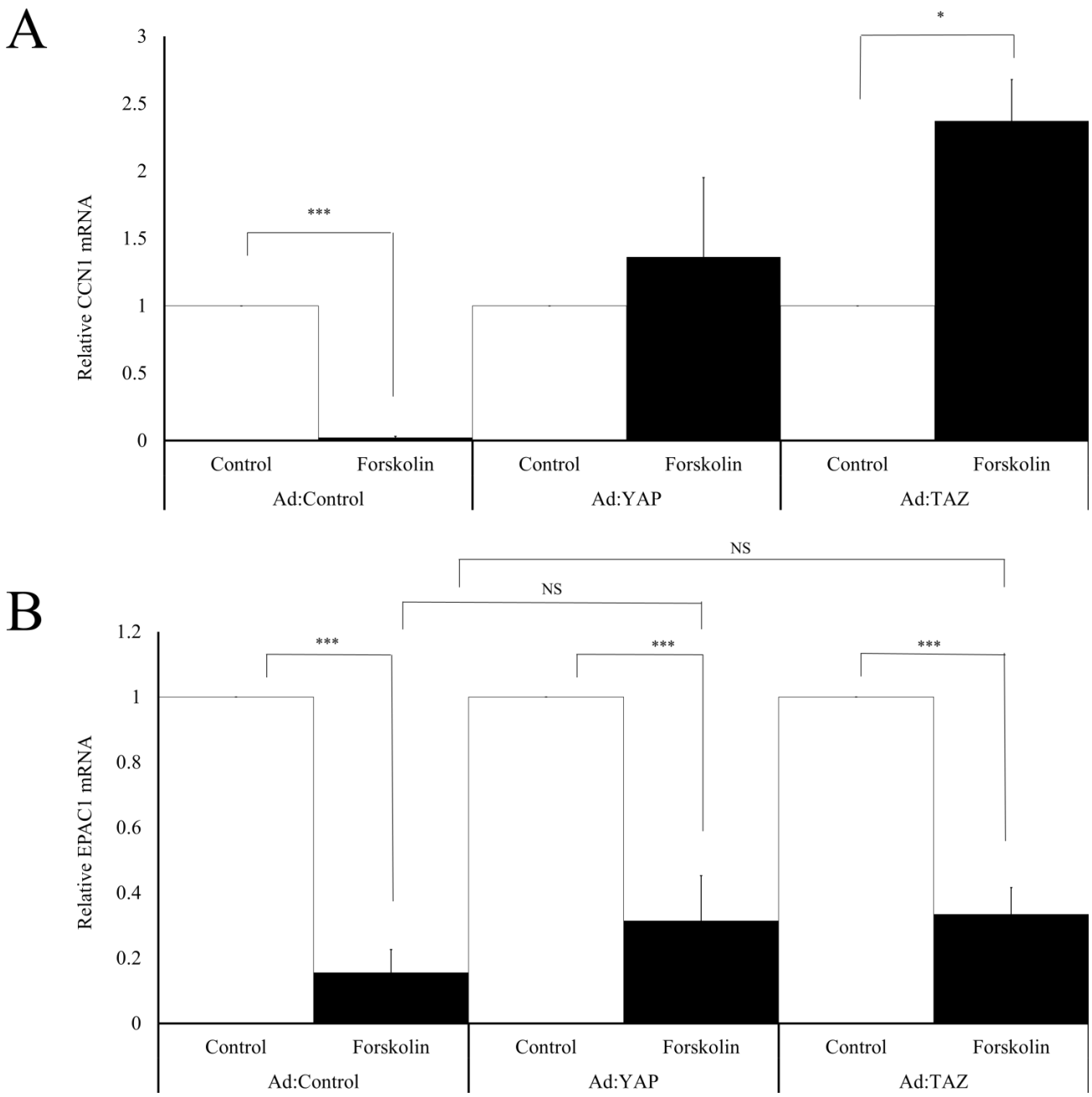


Figure 5.26: YAP and TAZ are insufficient for EPAC1 mRNA expression.

Rat cardiac fibroblasts were transfected with TEAD-LUC (**A**) or EPAC-NLUC (**B**) reporter gene plasmids. The next day, cells were infected with either a control adenovirus (Ad:Control) or adenovirus expressing constitutively-active YAP or TAZ, as indicated (A-D). Cells were then stimulated with 25 μM forskolin for 8 hours and reporter gene activity quantified (A and B) or total RNA analysed by qRT-PCR for EPAC1 mRNA or CCN1 mRNA levels as indicated. (C and D). *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=3.

5.3.6 HDAC1/3-mediated deacetylation of histone-3 lysine 27 contributes to cAMP-mediated inhibition of EPAC1 expression

The regulation of endogenous gene expression requires transcription factor binding to the gene's promoter region in addition to epigenetic remodelling of the local chromatin organisation. In contrast, plasmid-based reporter gene vectors are not usually packaged with histone proteins into chromatin and are largely regulated by transcription factor activity alone. As a result of this, we studied the possibility of epigenetic control in cAMP-mediated repression of EPAC1 expression. Since acetylation of histone-3 at lysinse-27 is associated with an open chromatin conformation and active gene expression, we specifically asked whether epigenetic regulation of histone3 lysine-27 acetylation is necessary, in addition to YAP-TEAD activity, for maximal EPAC1 expression. We tested the hypothesis that changes in histone 3 acetylation in the vicinity of the endogenous EPAC1 promoter is involved in the repression of EPAC1 mRNA expression in response to elevated cAMP. Moreover, we asked if the necessity of histone-3 lysine-27 (H3K27) acetylation explains inability of YAP or TAZ over expression to rescue EPAC1 mRNA levels in forskolin stimulated cardiac fibroblasts. In order to test this, chromatin immunoprecipitation (ChIP) was used to quantify the effects of cAMP elevation on H3K27 acetylation at the proximal EPAC1 promoter.

H3K27 acetylation was detected at the EPAC1 proximal promoter region in cultures of cardiac fibroblasts. Also, forskolin stimulation of cardiac fibroblasts resulted in a significant reduction in H3K27 acetylation levels at this region (Figure 5.27A). In contrast, levels of H3K27 acetylation at the proximal promoter of HAS1, a CREB-target gene that is induced by cAMP, were significantly increased following forskolin stimulation. This suggests that changes induced by forskolin in H3K27 acetylation, are region-specific and do not reflect global changes in H3K27 acetylation levels. Notably, the inhibitory effects of forskolin on H3K27 acetylation at the EPAC1 promoter was partly but significantly rescued by pre-stimulation of cardiac fibroblasts with the HDAC1/3-selective HDAC inhibitor (MS-275), which was not the case for CREB-target gene, HAS1, yet again showing the specificity in H3K27 acetylation mediated by HDAC1/3 (Figure 5.27B). We therefore tested if HDAC1/3 inhibition would cooperate with YAP overexpression to rescue forskolin-induced inhibition of EPAC1 mRNA. In the control group, forskolin treatment, significantly inhibited EPAC1 mRNA levels (Figure 5.28A). The inhibitory effects of forskolin were not rescued by YAP_{S127A} over expression (Figure 5.28A). This is consistent with the data in Figure 5.28B. By contrast, HDAC inhibiton partially reverse the inhibitory effects induced by forskolin on EPAC1 mRNA. Importantly, however, a combination of MS-275 and YAP_{S127A} expression completely prevented forskolin-mediated inhibition of EPAC1 mRNA levels, whereas

expression of the housekeeping gene 36B4 remained unaffected by any of the treatments (Figure 5.23B). Hence, H3K27-acetylation appeared to facilitate TEAD dependent transcription of EPAC1 and cAMP elevation triggered H3K27-deacetylation as well as blocking TEAD activity.

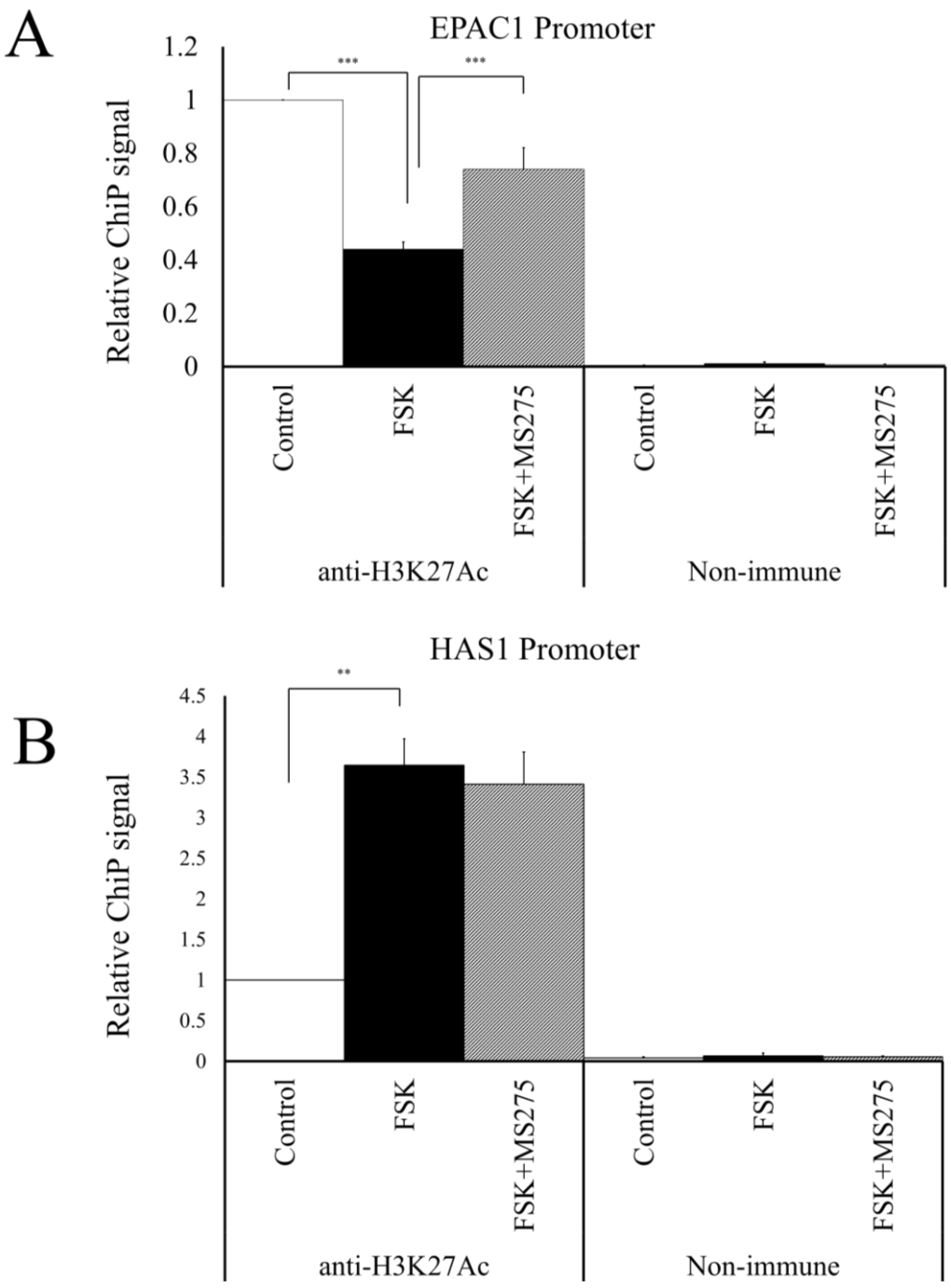


Figure 5.27. Forskolin deacetylates histone-3 lysine-27.

Rat cardiac fibroblasts were treated with 5 µM MS275 for 18 hours followed by stimulation with 25 µM forskolin for a further 2 hours (**A** and **B**). Cells were fixed and chromatin extracted and analysed for H3K27 acetylation at the proximal EPAC1 (**A**) or HAS1 (**B**) promoter regions. **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

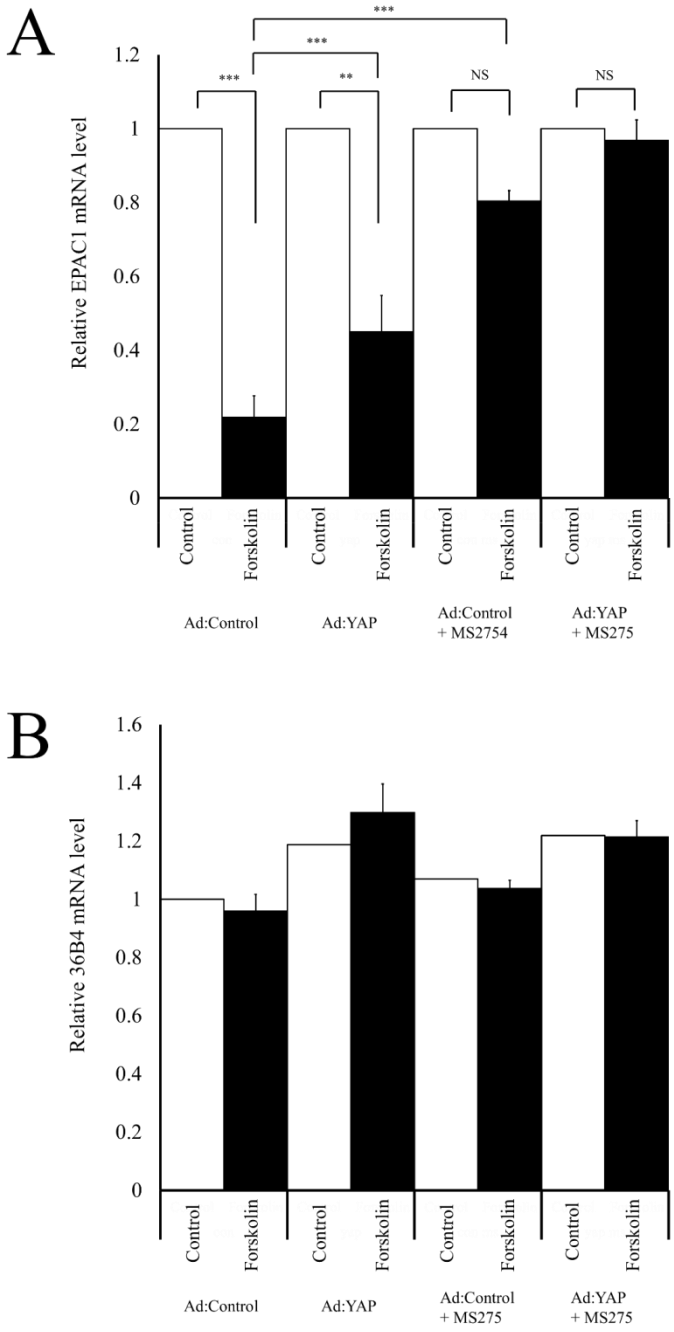


Figure 5.28: HDAC1/3 mediated deacetylation of histone-3 lysine-27 contributes to cAMP-mediated inhibition of EPAC1 mRNA expression.

Rat cardiac fibroblasts were infected with control adenovirus (Ad:Control) or adenovirus expressing constitutively active YAP (Ad:YAP) (**A** and **B**). The next day, cells were treated with 5 µM MS275 for 18 hours followed by stimulation with 25 µM forskolin for a further 2 hours. Total RNA was extracted and analysed by RT-qPCR for EPAC1 (**A**) or 36B4 (**B**) mRNA levels. **: $p<0.01$ and ***: $p<0.001$. Data are expressed as mean ±SEM and analysed by ANOVA with Student Newman Keul's post-test, n=4.

5.3.7 DISCUSSION

A large body of literature documents the ability of cAMP to activate EPAC signalling (de Rooij, Zwartkruis et al. 1998, de Rooij, Rehmann et al. 2000, Bos 2006, Hewer, Sala-Newby et al. 2011). However, the mechanisms responsible for regulating the expression of the EPAC gene is unknown. Here, we studied the mechanism responsible for the regulation of EPAC1 gene expression in cardiac fibroblast in normal conditions and in response to cAMP elevating stimuli. We documented that protein and mRNA levels of EPAC1 are regulated by TEAD-dependent transcription and that H3K27 acetylation is essential for TEAD activity at the EPAC proximal promoter.

In this chapter, it was demonstrated that elevated intracellular levels of cAMP rapidly suppress the protein and mRNA levels of EPAC1 via two distinctive signalling mechanisms. The first mechanism involves depolymerisation of actin cytoskeleton in response to cAMP, which results in the subsequent inactivation of YAP/TAZ-TEAD transcription factor activity. The second mechanism involves a cAMP-induced deacetylation of H3K27 in the vicinity of the endogenous EPAC1 promoter. Inhibition of EPAC1 expression via these mechanisms diminishes EPAC1 signalling and EPAC1-dependent morphological changes in response to subsequent cAMP-elevating stimuli. Together, these mechanisms represent a newly discovered system of crosstalk that controls EPAC1 expression in response to cAMP elevating stimuli and is crucial in regulating cellular responses to cAMP elevation (Figure 5.29).

In detail, elevating intracellular concentrations of cAMP, using either forskolin, cAMP analogues or physiological agonists of the A2BR, down regulate EPAC1 expression in cardiac fibroblasts. Specific activation of PKA or EPAC was able to suppress the mRNA levels of EPAC1 and activated together they acted additively to reduce EPAC1 expression. Interestingly, specific stimulation of PKA was more effective compared to specific EPAC activation at down regulating mRNA levels of EPAC1, suggesting a dominant role for PKA-mediated negative crosstalk in controlling EPAC1 expression. Negative crosstalk between PKA and EPAC have been reported previously, where PKA activation can oppose EPAC induced phosphorylation of AKT (Mei, Qiao et al. 2002). Moreover, PKA can antagonise the ability of the EPAC effector protein (Rap1) to form new blood vessels (Menon, Doebele et al. 2012). PKA can also regulate cAMP-dependent activation of EPAC by controlling the availability of cAMP via PDEs (Baillie, Scott et al. 2005). The novel findings presented here show that activation of PKA and EPAC play crucial roles in controlling the mRNA levels of EPAC1 in cardiac fibroblasts.

Inhibition of EPAC1 expression in cardiac fibroblasts in response to cAMP elevating stimuli is functionally linked with acquisition of a stellate morphology, which is characterised by a condensed cytoplasm, extended membrane protrusion and loss of F-actin stress fibres. Morphological changes and actin depolymerisation induced by cAMP elevation have been described in some other cell types, for example, smooth muscle cells (Hewer, Sala-Newby et al. 2011, Duggirala, Kimura et al. 2015, Smith, Hudson et al. 2017), astrocytes (Hatten 1985) and mesenchymal stem cells (Zhang, Yun et al. 2011). Importantly, in cardiac fibroblasts, the magnitude of the stellate morphology and reduction in actin-cytoskeleton polymerisation induced by activation of PKA and EPAC reflects the repression of EPAC1 gene expression. However, cAMP-induced actin remodelling, and inhibition of actin polymerisation are cell-type specific. Even though, cAMP elevating stimuli causes pronounced actin-cytoskeleton disruption in cardiac fibroblasts and smooth muscle cells (Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014), the similar scenario is not repeated in endothelial cells, where cAMP signalling promotes cortical actin polymerisation via PKA and EPAC activation (Cullere, Shaw et al. 2005, Birukova, Zagranichnaya et al. 2007, Prasain and Stevens 2009). In addition to this, as demonstrated here, elevation of cAMP levels do not cause morphological changes or actin depolymerisation in H9C2 embryonic cardiomyocytes cell line. Notably, EPAC1 expression is not suppressed by cAMP signalling in these cells, indicating that downregulation of EPAC1 mRNA levels, at least in part, is mediated by these changes in the actin-cytoskeleton polymerisation status. Hand in hand with this, we showed that, indirect actin depolymerisation, using the ROCK inhibitor Y27632, or directly targeting actin filaments with the actin-binding agents cytochalasin-D or latrunculin-B, also retards EPAC1 expression in cardiac fibroblasts. To our knowledge, this is the first time that EPAC1 expression has been linked to actin-cytoskeleton remodelling. Our new results confirm the existence of a novel negative crosstalk mechanism where PKA cooperates with EPAC activity to induce actin-cytoskeleton remodelling, and this mediates the suppression of EPAC1 gene expression.

Previous studies have reported the association between different signals with EPAC1 expression. For example: TGF-β stimulation can have stimulatory or repressive effects on EPAC1 expression in cardiac fibroblasts or myofibroblasts respectively (Olmedo, Munoz et al. 2013). Studies in rodents demonstrated that, prostaglandin stimulation reduces EPAC1 protein levels in liver fibrosis (Schippers, Beljaars et al. 2017). Stimulation of beta-adrenoreceptors with isoproterenol up regulated the expression of EPAC in atrial fibroblasts (Surinkaew, Aflaki et al. 2019). On the other hand, the responsible mechanisms that control EPAC1 gene expression in response to physiological signals are not yet clear.

Here we demonstrate a feedback mechanism that down regulate EPAC1 gene transcription in response to cAMP elevating stimuli. We demonstrate that levels of pre-spliced EPAC1 mRNA, (Elferink and Reiners 1996), are rapidly down regulated by cAMP-elevating stimuli. Furthermore, the activity of the proximal EPAC1 promoter region is also suppressed by these cAMP stimuli, which further proves the transcriptional suppression of EPAC1. By analysing the EPAC1 proximal promoter region, we found a conserved binding element for the TEAD transcription factors. Crucially, the activity of TEAD depends on actin-cytoskeleton organisation and integrity (Dupont, Morsut et al. 2011). Consistent with our findings showing that the gene expression of EPAC1 is also dependent on actin-cytoskeleton. Here, we demonstrate that cAMP elevating stimuli down regulate TEAD activity in cardiac fibroblasts, which is associated with increased phosphorylation of YAP and a reduced levels of total YAP protein in the nucleus and cytoplasm.

Also, we demonstrate that, YAP/TAZ-TEAD activities are needed for EPAC1 gene expression. For example, inhibition of TEAD activity using silencing of the TEAD co-factors YAP and TAZ, overexpression of a dominant-negative TEAD mutant or pharmacological inhibition of TEAD activity, all significantly down regulate the transcription of EPAC1. To our knowledge, this is the first time that EPAC1 expression has been linked to cytoskeleton integrity and YAP/TAZ-TEAD activity.

Even though we reported a clear need for YAP/TAZ-TEAD activity for EPAC1 expression, our data show that activation of this mechanism on its own, via YAP/TAZ over expression, is not enough to rescue cAMP-induced suppression of EPAC1 gene expression. This emphasis the need for the presence of other mechanisms. Although, a complete rescue of cAMP-induced inhibition of EPAC1 promoter activity was observed following the over expression of constitutively active YAP/TAZ, but their over expression did not rescue the down regulation of EPAC mRNA levels.

Since plasmid-based reporter gene vectors are not packaged with histone proteins into chromatin, we postulated that cAMP-induced down regulation of EPAC1 expression could also be mediated by epigenetic regulations and modifications on the histones. Hand in hand with this, a significant decrease was observed in H3K27 acetylation, an epigenetic modification associated with active transcription (Heintzman, Stuart et al. 2007), at the proximal EPAC1 promoter region in cardiac fibroblasts stimulated with forskolin. Our findings suggest that this loss of H3K27 acetylation was mediated by either HDAC1 or HDCA3, since loss of H3K27 acetylation at the EPAC1 promoter following forskolin stimulation was reversed by the HDAC1/3 selective inhibitor (MS-275). Moreover, our findings suggest that this epigenetic modification acts simultaneously with the

inhibition of YAP/TAZ-TEAD binding to regulate EPAC1 expression following cAMP stimulation. For instance, inhibition of HDACs1/3 or YAP/TAZ over expression alone, only partially reversed the inhibitory effects of forskolin on the mRNA levels of EPAC1 in cardiac fibroblasts. On the other hand, HDACs1/3 inhibition together with constitutively active YAP/TAZ completely reversed the inhibitory effects of forskolin.

In summary, our data demonstrate that inhibition of YAP/TAZ-TEAD activity and HDACs1/3 -mediated H3K27 deacetylation are functionally important in the suppression of EPAC1 expression in cardiac fibroblasts following cAMP stimulation. We suggest that these two mechanisms represent a novel mechanism of cross talk that controls EPAC1 signalling. These feedback cycles are found in almost all signalling pathways, functioning to maintain normal physiological signalling homeostasis and allowing for adaptive cellular responses to a continuous signal. Suppression of EPAC1 expression via the cycle shown here is likely to be important in the temporal regulation of EPAC1 signalling in cardiac fibroblasts, which produce temporary signal responses in prolonged cAMP elevation. Specific down regulation of EPAC1 but not PKA catalytic subunits indicate that subsequent cAMP signalling may also be adapted towards PKA-specific responses.

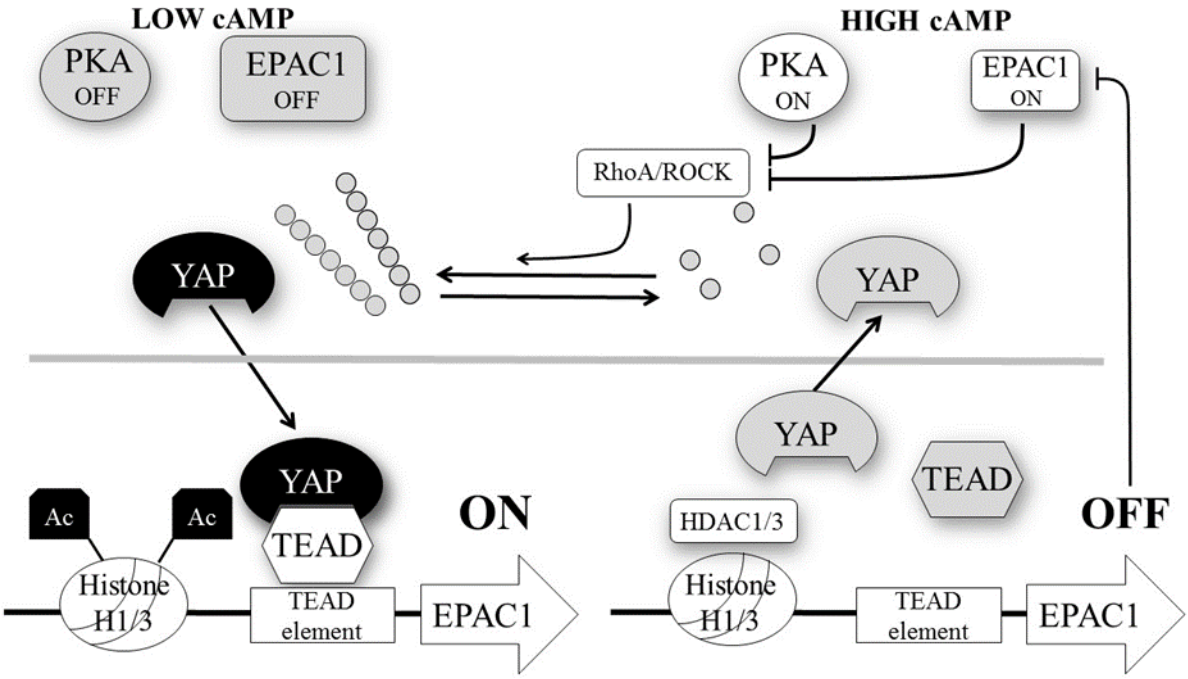


Figure 5.29: Proposed mechanism underlying cAMP-dependent repression of EPAC1 expression.

In cardiac fibroblast with low levels of cAMP, PKA and EPAC activity are low and RhoA/ROCK activity is high. This results in actin polymerisation and nuclear translocation of YAP/TAZ. YAP/TAZ increase activity of TEAD transcription factors, which act via a conserved TEAD binding element in the proximal EPAC1 promoter to induce EPAC1 gene expression. Elevated cAMP levels activate PKA and EPAC1, which cooperate to inhibit RhoA/ROCK-mediated actin polymerisation, resulting in reduced actin polymerisation and acquisition of a stellate cell morphology. This induces nuclear export of YAP/TAZ, which in turn reduces TEAD activity. Elevated cAMP also induces a HDAC1/3-mediated reduction in histone H3K27 acetylation at the proximal EPAC1 promoter. The reduction in TEAD activity together with the reduction in histone H3K27 acetylation inhibits EPAC1 gene expression.

CHAPTER 6:

GENERAL DISCUSSION

6.1 DISCUSSION

Aberrant proliferation and migration of cardiac fibroblasts is a major factor that contributes toward the development of cardiac fibrosis and ultimately heart failure, which is collectively the leading causes of global death in developed countries (Travers, Kamal et al. 2016, Humeres and Frangogiannis 2019, Yuan, Pan et al. 2019). To date, there are only limited anti-cardiac fibrosis therapies available to patients. Although several anti-fibrotic therapies have been studied in animal models of cardiac fibrosis, with some providing encouraging results, translating these promising findings into effective therapies for human patients has been challenging. Clinical trials of some therapies, such as the use of the phosphodiesterase inhibitor Milrinone, produced disappointing results, with long-term therapy actually being associated with increased morbidity and mortality in patients suffering from several chronic heart failure (Packer, Carver et al. 1991). Therefore, it is clear that new therapeutic strategies are needed to effectively limit the development of cardiac fibrosis and its progression to heart failure. To achieve this, it is essential to have a detail understanding of the underlying mechanisms the regulate the development of cardiac fibrosis.

Several processes contribute towards the development of cardiac fibrosis, including, chronic inflammation (Nicoletti and Michel 1999, Suthahar, Meijers et al. 2017), ECM deposition (Kong, Christia et al. 2014, Suthahar, Meijers et al. 2017), pressure overload (Rodriguez-Pascual and Diez 2017, Goh, He et al. 2019) and increased cardiac fibroblasts proliferation (Travers, Kamal et al. 2016, Humeres and Frangogiannis 2019). Although many studies have highlighted and demonstrated the importance of up regulation in ECM gene expression, increases in the number of ECM producing cardiac fibroblasts through proliferation is also an important factor. Therefore, a better understanding of the mechanisms that normally regulate cardiac fibroblasts proliferation may help identify novel therapeutic targets which could be exploited to limit the progression of cardiac fibrosis.

An important characteristic of cardiac failure is irregular second messenger signalling due to abnormal synthesis and metabolism of cAMP (Efendiev and Dessauer 2011, Boularan and Gales 2015, Kim and Kass 2017). In this sense, the cAMP signalling pathway may therefore represent a promising therapeutic target. For example, cAMP inhibited the action and formation TGF-β and angiotensin II in rat hepatic fibroblasts (Windmeier and Gressner 1997), rat kidney fibroblasts (Kothapalli, Hayashi et al. 1998), human renal fibroblasts (Heusinger-Ribeiro, Eberlein et al. 2001) and human gingival fibroblasts (Black, Palamakumbura et al. 2007). Likewise, elevated levels of cAMP have been shown to have anti-cardiac fibrotic effects in experimental animal models of cardiac fibrosis, including suppression of collagen production and inhibition of cardiac fibroblasts

proliferation (Dubey, Gillespie et al. 2001, Liu, Ostrom et al. 2004, Ryzhov, Sung et al. 2014). For example, experiments in rats demonstrated that stimulation of A2BRs reduced tissue scar formation (Ryzhov, Sung et al. 2014). Consistent with this, silencing of the A2BRs up regulated collagen synthesis and the proliferation of cardiac fibroblast, whereas their overexpression demonstrated opposing results (Dubey, Gillespie et al. 2001). Some of these protective properties of cAMP may be mediated, at least in part via suppression of cardiac fibroblast proliferation. For example, cAMP elevating stimuli are known to inhibit proliferation in several different cell types, such as, keratinocytes (Takahashi, Honma et al. 2004), thyroid carcinoma cell line (Rocha, Paternot et al. 2008), hepatoma cells (Van Meeteren, Loesberg et al. 1982) and vascular smooth muscle cells (Smith, Hudson et al. 2017). Moreover, several investigations have reported anti-cardiac fibrotic properties of PKA and EPAC signalling (Liu, Ostrom et al. 2004, Aroonsakool, Yokoyama et al. 2008, Yokoyama, Patel et al. 2008, Insel, Murray et al. 2012, Liu, Xu et al. 2017, Sivertsen Asrud, Pedersen et al. 2019). Interestingly, it was also demonstrated that EPAC expression is down regulated during cardiac fibrosis (Insel, Murray et al. 2012). Therefore, it is important to characterise the mechanism responsible for EPAC down regulation in cardiac fibroblasts.

Translating the anti-cardiac fibrotic of cAMP into clinical practise has been challenging. Direct delivery of cAMP analogues can be problematic due to poor cell permeability and stability. Moreover, elevating the levels of cAMP by inhibiting PDEs is associated with increased mortality in long term treatment (DiBianco 1989, Amsallem, Kasparian et al. 2005). Therefore, there is a need to characterise specific downstream cAMP signalling pathways to target therapeutically, rather than global disruption of the cAMP signalling system.

One of the aims if this thesis was to characterise the regulation cardiac fibroblast proliferation by cAMP and the role played by the MKL1-SRF and YAP/TAZ-TEAD transcripfactor:co-factor complexes. We showed that cAMP-induced actin-depolymerisation inhibits mitogen-induced nuclear localisation of MKL1 and YAP in cardiac fibroblasts and this mechanism underlies, at least in part, the anti-proliferative (but not anti-migratory) effects of cAMP in these cells. In detail, elevation of cAMP levels in cardiac fibroblasts using either A2BR agonist, forskolin or Db-cAMP analogue rapidly down regulated Rho/ROCK signalling, suppressed actin-polymerisation (F-actin) levels, increased actin-depolymerisation (G-actin) levels and completely inhibited mitogen-induced nuclear localisation of MKL1 and reduced YAP nuclear localisation and levels. These anti-mitogenic effects of cAMP were mediated by both PKA and EPAC, that acted in a cooperative manner and were associated with a change in cell morphology and reduction in actin polymerisation. This cAMP mediated inhibition of actin polymerisation was likely mediated by inhibition of RhoA-

ROCK signalling since it was associated with reduced ROCK phosphorylation and pharmacological inhibition of ROCK reduced similarly inhibited actin polymerisation and cardiac fibroblast proliferation.

The anti-proliferative effects of cAMP signalling demonstrated here are in consistent with those reported previously in vascular smooth muscle cells and other cells types (Hayashi, Morishita et al. 2000, Growcott, Spink et al. 2006, Rocha, Paternot et al. 2008, Smith, Hudson et al. 2017, Smith, Sessions et al. 2019). Previous studies in our lab showed that, cAMP elevation inhibits the proliferation of vascular smooth muscle cells by inhibiting entry into S-phase of the cell cycle via a mechanism that requires the activation of PKA and EPAC (Smith, Hudson et al. 2017). Using PKA and EPAC selective agonists, data presented here, show the necessity of PKA and EPAC activation. However, there are some key differences between the anti-mitogenic effects of PKA and EPAC signalling in cardiac fibroblasts and those previously reported in vascular smooth muscle cells. Selective activation of either PKA or EPAC alone did not inhibit serum-induced proliferation vascular smooth muscle cells (Hewer, Sala-Newby et al. 2011). This contrasts with the data presented here, which shows a significant but submaximal down regulation of proliferation in cardiac fibroblasts. In addition, PKA and EPAC activation acted synergistically to down regulate the proliferation of vascular smooth muscle cells, whereas the inhibitory effects of PKA and EPAC on the proliferation of cardiac fibroblasts were only additive. Interestingly, our data is also consistent with work published by Yokoyama *et al* showing that selective EPAC activation had anti-proliferative effects in rat smooth muscle cells (Yokoyama, Minamisawa et al. 2008). To add to the complexity, the effects of cAMP/PKA/EPAC signalling pathways on proliferation are variable depending on the cell types. For example, abundant published evidence reports the anti-mitogenic properties of cAMP in vascular smooth muscle cells (Hewer, Sala-Newby et al. 2011, Kimura, Duggirala et al. 2014, Smith, Hudson et al. 2017). However, there are studies demonstrating that cAMP signalling can promote the prolfieration of endothelial cells (Smith, Hudson et al. 2017) epithelial cells (Yamaguchi, Nagao et al. 2003), embryonic stem cells (Lee, Kim et al. 2012) and even VSMC under some conditions (Hogarth, Sandbo et al. 2004). The reasons for different effects of cAMP on proliferation are not fully understood but may reflect differences in the relative expression levels of PKA and/or EPAC in these cell types or a variation in how these two cAMP effectors mediate downstream signalling pathways, including the actin remodelling mechanisms that have been implicated in mediating cAMP-dependent anti-mitogenesis (Bond, Wu et al. 2008, Hewer, Sala-Newby et al. 2011, Parnell, McElroy et al. 2017).

The data here also shows that, pharmacological inhibition of ROCK with Y27632, depolymerises the actin filaments, induces morphological changes and down regulates the proliferation of cardiac fibroblasts. Our findings are again consistent with the effects of ROCK inhibition on vascular smooth muscle cells, where Y27632 induced similar effects. However, this is in contrast with other investigations where Y27632 promoted the proliferation of embryonic stem cells (Croze, Thi et al. 2016), spinal cord astrocytes (Yu, Liu et al. 2012), keratin producing epidermal cells (Chapman, McDermott et al. 2014) and mouse embryonic fibroblasts (Kumper, Mardakheh et al. 2016). It has been suggested that, Y27632 promotes astrocytes proliferation via up regulating the protein levels of CDKs, therefore promoting the progression of cell cycle (Yu, Liu et al. 2012). Also it has been demonstrated that, Y27632 upregulated the genes involved with cell division and biosynthesis, such as, *Crct1*, *Flg* and *Ivl*, again promoting cell mitosis (Chapman, McDermott et al. 2014). Although, we did not check the effect of Y27632 on the protein and mRNA levels of CDKs or genes involved in cell division, but our results suggest that Y27632 down regulated the SRF and TEAD target genes, regulating proliferation, such as, *Ccn1* and *Ctgf*.

While cAMP-induced actin-depolymerisation down regulated the proliferation of cardiac fibroblasts, the migration of these cells was not affected. In some cases, elevated levels of cAMP (via Db-cAMP stimulation) and pharmacological ROCK inhibition even stimulated the migration of cardiac fibroblasts in the experiments presented here. Even though these stimulatory effects were modest, they contrasted with the large inhibitory effects of cAMP previously reported in vascular smooth muscle cells by our group (Smith, Hudson et al. 2017) and others (Yokoyama, Minamisawa et al. 2008, Hayashi, Murai et al. 2015, McKean, Murray et al. 2015, Parnell, McElroy et al. 2017, Smith, Hudson et al. 2017). This difference suggests the existence of a distinct signalling mechanisms involved in the regulation of proliferation and migration in these cells. In vascular smooth muscle cells, similar mechanisms have been implicated in the cAMP-dependent regulation of both proliferation and migration. For example, elevated levels of cAMP in vascular smooth muscle cells inhibits the activity of the actin-sensitive transcription factor:co-transcription factor complexes MKL-SRF and YAP/TAZ-TEAD, both of which have been shown to play a role in the regulation of vascular smooth muscle cells proliferation and migration (Johnson and Walker 1999, Smith, Hudson et al. 2017, Smith, Sessions et al. 2019). The regulation of MKL-SRF and YAP/TAZ-TEAD by cAMP stimulation in cardiac fibroblasts is presented in chapters 4 and 5 of this thesis where it is shown how they are negatively regulated by cAMP, consistent with previous work in vascular smooth muscle cells (Kimura, Duggirala et al. 2016, Parnell, McElroy et al. 2017). Despite cAMP-elevating stimuli clearly inhibiting the activity of MKL-SRF and YAP-TEAD in the

experiments presented here, the migration of these cells was not inhibited. This clearly implies that cardiac fibroblasts are able to migrate independently of MKL-SRF and YAP/TAZ-TEAD activity.

The requirement of actin remodelling for cells to migrate is widely accepted. Cell migration requires continuous dynamic reorganisation of the actin, including increased actin polymerisation to promote lamellipodia formation and strengthen new focal adhesions at the leading edge but at the same time requires actin depolymerisation and focal adhesion disassembly at the rear, allowing for retraction of the trailing edge driven by actomyosin contractility, as explained in chapter 1 of this thesis (Pollard and Cooper 2009). Rho GTPase activity plays a key role in promoting actin polymerisation at the leading edge and is essential for migration of cardiac fibroblasts in two-dimensional migration assays (Jatho, Hartmann et al. 2015). Silencing of RhoA in cardiac fibroblasts inhibits their migration in 2D scratch wound assay (Jatho, Hartmann et al. 2015), consistent with a requirement for RhoA-mediated actin polymerisation. On the other hand, genetic silencing of RhoA increases the migration of cardiac fibroblast through a three-dimensional collagen gel by allowing and promoting an amoeboid mode of migration (Jatho, Hartmann et al. 2015). This indicates that cardiac fibroblasts use two mechanistically different modes to migrate, a RhoA and actin-dependent mesenchymal mode of migration and a RhoA and actin independent amoeboid mode of migration. The data presented here demonstrates that cAMP elevation with forskolin, Db-cAMP analogue or Y27632 does not inhibit cardiac fibroblast migration despite clear inhibitory effects on actin polymerisation. This suggests that cardiac fibroblasts adopt an amoeboid mode of migration under conditions of elevated cAMP and impaired actin polymerisation. Yokoyama *et al* also reported enhanced cardiac fibroblast migration in response to elevated cAMP. However, this enhancement was only evident with low but not high levels of cAMP (Yokoyama, Patel et al. 2008). The inhibitory effects of higher concentrations of forskolin reported by Yokoyama *et al* may be due to migrations assays being performed under serum free conditions, whereas, our migration assays were all performed in the presence of serum. Serum stimulates the activity of RhoGTPase suggesting that these differences may reflect the magnitude of inhibition of RhoGTPase activity and actin polymerisation. Here the data demonstrate forskolin stimulation reduces the percentage of F-actin from 40% to 30%. Notably, similar analyses in vascular smooth muscle cells demonstrate a much larger reduction in F-actin content in response to forskolin stimulation (from 57% to 9%). This larger inhibition of actin polymerisation by cAMP in vascular smooth muscle cells may explain the divergent effects of cAMP in the two different cell types. Although the physiological significance of the divergent effects of cAMP on cardiac fibroblast migration is currently unknown, the ability of cardiac fibroblast to migrate under conditions of elevated cAMP and impaired actin

polymerisation, possibly by adopting an amoeboid mode of migration, may highlight the critical importance of rapid cardiac repair after acute myocardial injury, such as following MI.

Various studies have demonstrated the controversial role of ROCK on cellular migration. Some investigations demonstrated the inhibitory roles of ROCK inhibition on migration (Yarrow, Totsukawa et al. 2005, Mikami, Yoshida et al. 2015, Sadok, McCarthy et al. 2015, Wang, Yang et al. 2016, Guerra, Oliveira et al. 2017, Smith, Hudson et al. 2017), whereas other showed that ROCK inhibition increases cellular mobility (Totsukawa, Wu et al. 2004, Yang and Kim 2014, Piltti, Varjosalo et al. 2015, Chang, Zhang et al. 2018). Here we showed that, even though Y27632 induced significant morphological changes, depolymerised the actin filament, downregulated the proliferation of cardiac fibroblasts and decreased the mRNA levels of SRF and TEAD target genes, but it enhanced the migration of these cells, which is in contrast to the investigations mentioned above. This may reflect cell type specific differences. It could also imply that cardiac fibroblasts implement an amoeboid mode of migration under conditions when ROCK is inhibited or their actin filaments are depolymerised, once more, reflecting the significance of rapid cardiac repair after MI, to prevent myocardium rupture.

Consistent with previous investigations in vascular smooth muscle cells, where it was reported that MKL1 localisation is regulated by cAMP (Smith, Hudson et al. 2017), here we also showed that cAMP elevating stimuli enhance the cytoplasmic localisation of MKL1 in cardiac fibroblasts (mediated by PKA and EPAC specific activation), down regulated the activities of SRF and TEAD reporter genes and suppressed the transcription of SRF and TEAD-target gene. Our data are also consistent with those reported in skin fibrosis where nuclear MKL1 expression was higher in fibrotic model compared to the healthy model (Shiwen, Stratton et al. 2015) and skin fibrosis was reduced in MKL1 knockout mice. Moreover, we demonstrated that pharmacological inhibition or genetic silencing of MKL, both exerted anti-proliferative effects in cardiac fibroblasts which is again consistent with those reported in vascular smooth muscle cells (Smith, Hudson et al. 2017) and prostate cancer cells (Evelyn, Wade et al. 2007).

Numerous studies have characterised the ability of cAMP to stimulate EPAC activity and established important physiological and pathological functions of EPAC activity and signalling (de Rooij, Zwartkruis et al. 1998, de Rooij, Rehmann et al. 2000, Bos 2006, Hewer, Sala-Newby et al. 2011). In chapters 3 and 4 of thesis, these functions were extended to characterise the role of EPAC signalling activity in regulating cardiac fibroblast proliferation and the regulation of the SRF co-factor MKL1. However, very little is known about the regulation of EPAC1 protein levels and the

expression of the EPAC1 gene. In chapter 5, data is presented that characterises a novel regulatory mechanism that controls the expression of the EPAC1 gene in cardiac fibroblasts in response to changes in the levels of cAMP. The data presented demonstrates that EPAC1 mRNA and protein expression are repressed by elevated cAMP levels. At the transcriptional levels, data is presented that demonstrates that EPAC1 transcription is dependent on the activity of the YAP/TAZ-TEAD transcriptional complex. In addition to the regulation by YAP/TAZ-TEAD, data is also presented that demonstrates that EPAC1 expression is also controlled by Histone H3-K27-acetylation at the proximal promoter region. Taken together, the data presented demonstrates that elevated intracellular cAMP rapidly represses the expression of EPAC1 mRNA and protein levels by antagonising both of these regulatory processes. In this way, we show that EPAC1 expression is sensitive to changes in the organisation of the actin cytoskeleton, via YAP/TAZ-TEAD. The second epigenetic mechanisms involve cAMP-mediated deacetylation of H3K27 at the EPAC1 proximal promoter region, which presumable controls chromatin accessibility and access of the YAP/TAZ-TEAD complex to the TEAD element located within the EPAC1 proximal promoter. Together, these mechanisms constitute a novel negative feedback mechanism that represses EPAC1 expression in response to cAMP elevating stimuli.

In more detail, cAMP elevation in cardiac fibroblasts using either forskolin, cAMP-analogues or physiological agonists of the A2BR rapidly represses EPAC1 expression in cardiac fibroblasts, suggesting that EPAC1 is sensitive to physiological changes in cAMP levels. This repression was mediated via signalling via both PKA and EPAC since selective activation or either EPAC or PKA alone inhibited EPAC1 mRNA expression, with simultaneous activation of PKA and EPAC acting additively. It is interesting to note that PKA activation was more effective than EPAC activation at down regulating EPAC1 expression. Firstly, this suggests the existence of a negative crosstalk between PKA and EPAC1. Similar negative-cross talk between PKA and EPAC1 signalling has been reported previously in other cell types. For example, PKA activity antagonises EPAC induced phosphorylation of AKT (Mei, Qiao et al. 2002). PKA can reduce the ability of the EPAC effector protein Rap1 to regulate angiogenesis (Menon, Doebele et al. 2012). PKA can also regulate cAMP-dependent activation of EPAC by controlling the availability of cAMP via phosphodiesterases (Baillie, Scott et al. 2005). Secondly, the greater effectiveness of PKA compare to EPAC1 activation in repressing EPAC1 expression mirrors the effects of these cAMP sensors on actin remodelling and cell morphological change. This likely reflects the dependence of EPAC1 expression on integrity of the actin cytoskeleton. Repression of EPAC1 expression in cardiac fibroblasts in response to elevated cAMP is associated with acquisition of a condensed ‘stellate’ shaped cell

morphology characterised by a condensed cytoplasm, extended membrane protrusion and loss of F-actin stress fibres. These cAMP-dependent morphological changes and inhibition of actin-polymerisation have been described in several other cell types, including smooth muscle cells (Hewer, Sala-Newby et al. 2011, Smith, Hudson et al. 2017), astrocytes (Hatten 1985) and mesenchymal stem cells (Zhang, Seitz et al. 2011). Consistent with a role of actin remodelling in the regulation of EPAC1, the data presented demonstrates that the magnitude of stellate morphology and reduction in actin-cytoskeleton polymerisation induced by activation of PKA and EPAC in cardiac fibroblasts is mirrored by the repression of EPAC1 gene expression. However, it is intriguing to consider how EPAC1 activation alone represses EPAC1 expression, since we are unable to detect any morphological change, and by implication little actin remodelling, in response to EPAC1 activation alone. A similar lack of actin remodelling was reported in VSMC in response to selective EPAC1 activation (Smith, Hudson et al. 2017). This may suggest the involvement on an actin-independent mechanism controlled by EPAC1 signalling. This mechanism may be the same as that involved in controlling MKL1 nuclear localisation, a protein that is widely reported to be actin-sensitive, that in our experiments was also sensitive to EPAC1 activation, in the absence of any detectable morphological change. Cyclic-AMP-induced stellate morphological changes and inhibition of actin polymerisation are cell-type specific. Although elevated cAMP induces rapid and pronounced actin-cytoskeleton disruption in cardiac fibroblasts and smooth muscle cells, this does not occur in ECs (Smith, Teixeira et al. 2013) or as we show here, the H9C2 cardiac myocyte cell line. Correspondingly, we show that EPAC1 expression is not repressed by cAMP-elevating stimuli in the cardiomyocyte H9C2 cell line, further supporting the conclusion that that repression of EPAC1 expression in response to cAMP is, at least in part, mediated by changes in the actin-cytoskeleton polymerisation status. Consistent with this, we also demonstrated that disruption of actin-polymerisation, using the ROCK inhibitor Y26732, or directly with the actin-binding drugs cytochalasin-D or latrunculin-B, also repress EPAC1 expression. To our knowledge this is the first time that EPAC1 expression has been linked to actin-cytoskeleton remodelling, although previous reports have linked EPAC1 localisation and function to actin organisation. For example, EPAC1 interaction with the actin binding ERM (Ezrin, Radixin and Moesin) proteins has been implicated in localisation of EPAC1 to the plasma membrane cortical actin, where it participates in cAMP-dependent regulation of cell proliferation and cell spreading (Gloerich and Bos 2010, Hochbaum, Barila et al. 2011). Our new data now establishes the existence of a novel negative crosstalk mechanism where PKA cooperates with EPAC activity to induce actin-cytoskeleton remodelling, which contributes to repression of EPAC1 gene expression.

This novel regulatory mechanism likely forms part of an important homeostatic physiological mechanisms that regulates the level of actin polymerisation within cardiac fibroblasts and other cells that display cAMP-induced actin depolymerisation. Based on the data presented it is likely that EPAC1 expression will gradually increase in response to increases in RhoA activity and actin polymerisation increases within a cell. Increased levels of EPAC1, in cooperation with PKA, will then enhance the repression of RhoA signalling and actin remodelling. Likewise, if RhoA activity and actin-polymerisation is low within a cell, EPAC1 expression will be repressed, thus relieving cAMP-dependent inhibition of the RhoA pathway and allowing actin polymerisation to be increase. Since actin dynamics play a central role in the regulation of so many diverse cellular functions, not least cell proliferation, this physiological homeostatic mechanism is likely to be very important in controlling normal cell behaviour in response to a wide range of extracellular signals (Figure 6.1).

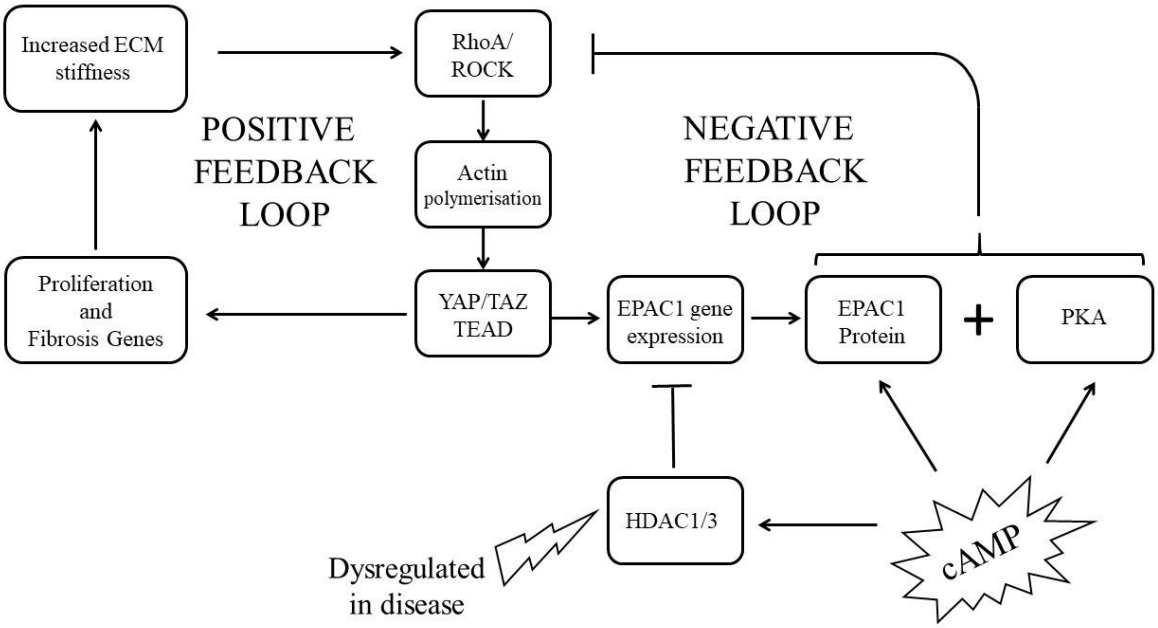


Figure 6.1: Schematic representation of positive and negative feedback loops regulating fibrosis and EPAC1 expression in cardiac fibroblasts

Rho-ROCK signalling promotes actin polymerisation, which enhances YAP-TEAD-dependent gene expression. Increased YAP-TEAD activity promotes EPAC1 transcription. EPAC1, in cooperation with PKA, inhibits Rho-ROCK activity, thus reducing actin polymerisation. This forms a negative feedback loop that helps maintain actin cytoskeleton homeostasis. HDACs are dysregulated in cardiac fibrosis. HDAC1/3 activity represses YAP-dependent EPAC1 expression, thus inactivating this negative feedback loop and allowing enhanced signalling through Rho-ROCK, actin polymerisation and YAP-TEAD activity. YAP-TEAD activity also promotes cardiac fibrosis and cardiac fibroblast proliferation. Tissue fibrosis increases the stiffness of the local ECM, which further enhances Rho-ROCK signalling. This forms a positive feedback loop that drive fibrosis.

Data is presented demonstrating that EPAC1 transcription is controlled by TEAD family transcription factors. Analysis of the EPAC1 promoter identified a conserved consensus TEAD binding element in the proximal promoter. Binding of recombinant TEAD protein to this site was confirmed by EMSA analysis and mutation of this element in the context of a luciferase reporter gene assay significantly reduced EPAC1 promoter activity. Importantly, TEAD transcription factor activity is known to be dependent on actin-cytoskeleton organisation and integrity (Dupont, Morsut et al. 2011), consistent with data presented showing that maximal EPAC1 expression is also dependent on actin-cytoskeleton integrity. Further confirming the importance of TEAD, data is presented demonstrating that maximal expression of the endogenous EPAC1 gene is dependent on YAP/TAZ-mediated TEAD activity. For example, inhibition of TEAD activity using silencing of the TEAD co-factors YAP and TAZ, overexpression of a dominant-negative TEAD mutant or inhibition of TEAD activity using two chemically distinct pharmacological inhibitors all significantly inhibited EPAC1 transcription. These data suggest that YAP/TAZ-TEAD is mechanistically responsible for coupling EPAC1 expression to the status of the actin cytoskeleton. In this way, enhanced YAP/TAZ-TEAD activity will promote EPAC1 expression that then acts in cooperation with PKA to promote actin de-polymerisation and in turn reduced YAP/TAZ-TEAD activity. To our knowledge, this is the first time that EPAC1 expression has been linked to cytoskeleton integrity and YAP/TAZ-TEAD activity. A large body of evidence reported the role played by YAP/TAZ-TEAD in sensing bio-mechanical signals (Kim, Kim et al. 2013, Das, Fischer et al. 2016, Dupont 2016, Meng, Moroishi et al. 2016, Elosegui-Artola, Andreu et al. 2017, Dobrokhotov, Samsonov et al. 2018, Boopathy and Hong 2019). This also implies that under condition of tissue fibrosis, which is typically associated with increased ECM stiffness and enhanced YAP/TAZ-TEAD activation (Dupont, Morsut et al. 2011, Nukuda, Sasaki et al. 2015, Noguchi, Saito et al. 2017, Noguchi, Saito et al. 2018), EPAC1 expression will be enhanced. Increases in EPAC1 expression have been reported in several pathological conditions. For example, EPAC1 levels are elevated during cardiac hypertrophy. EPAC1 levels are also increased after experiment angioplasty injury to the rat carotid artery. It will be important to determine in the future if activation of YAP/TAZ-TEAD during these pathological processes is involved in mediating the reported increases in EPAC1.

Although we demonstrate a clear requirement for YAP/TAZ-TEAD activity for EPAC1 expression, our data also demonstrates that activation of this mechanism in isolation, via expression of constitutively-active YAP or TAZ mutants, is insufficient for expression of EPAC1 mRNA, highlighting the involvement of additional mechanisms. While expression of constitutively-active

YAP or TAZ were able to completely reverse cAMP-dependent repression of the EPAC1 promoter driven reporter gene activity, they were not able to reverse the inhibition of EPAC1 mRNA levels. Since plasmid-based reporter gene vectors are not typically packaged with histone proteins into chromatin, we reasoned that histone-associated epigenetic regulation was also involved in cAMP-mediated repression of EPAC1 expression. Consistent with this, we detected a significant decrease in histone-3 lysine-27 acetylation (H3K27Ac), an epigenetic modification associated with active transcription (Heintzman, Stuart et al. 2007), at the proximal EPAC1 promoter region in cardiac fibroblasts stimulated with forskolin. Our data suggests that this loss of H3K27Ac was mediated by HDAC1 or HDCA3, since loss of H3K27Ac at the EPAC1 promoter in response to forskolin stimulation was reversed by the HDAC1/3 selective inhibitor MS-275. Importantly, our data demonstrates that this epigenetic modification acts cooperatively with inhibition of YAP/TAZ-TEAD to mediated cAMP-dependent repression of EPAC1 expression. For example, HDAC inhibition or expression of constitutively active YAP/TAZ alone only partially rescued EPAC1 mRNA expression in forskolin-stimulated cells, whereas HDAC inhibition together with constitutively-active YAP/TAZ fully reverse the inhibitory effects of forskolin.

Taken together, our data demonstrates that inhibition of YAP/TAZ-TEAD and HDAC1/3 -mediated H3K27-deacetylation underlies the repression of EPAC1 expression in cardiac fibroblasts in response to cAMP-elevating stimuli. We suggest that this mechanism represents a novel mechanism of cross talk that controls EPAC1 signalling. Such loops that feed an inverted output signal back to the input are found in nearly all known signalling pathways, serving to maintain normal physiological signalling homeostasis and allowing for adaptive cellular responses to a persistent signal. Down regulation of EPAC1 expression via the control loop we describe here is likely to be important in the temporal regulation of EPAC1 signalling, generating transient signals responses in prolonged cAMP-elevation. Selective down regulation of EPAC1 but not PKA catalytic subunits suggests that subsequent cAMP signalling may also be adapted towards PKA-specific response.

6.2 LIMITATIONS OF THIS RESEARCH

This study has potential limitations. Most of our experiments were carried out in rodents' cardiac fibroblasts, therefore we need more replications in human cardiac fibroblasts. Moreover, a lot of our experiments were performed with forskolin, which can induce supra-physiological levels of cAMP. Although some data is presented demonstrating similar effects of GPCR agonists that induce more physiological levels of cAMP, more research is needed to fully characterise the regulation of these mechanisms by physiological stimuli.

Additionally, our investigations did not test the regulation or function of MKL2 compared to MKL1. Therefore, future investigations need to be carried out in order to address this.

Also, our work did not test the role of specific TEAD isoforms or the role of different TEAD transcriptional coactivator. Considering the existence of different TEAD isoforms and transcriptional coactivators, it would be important to investigate the currently unidentified roles of each TEAD isoforms and each TEAD transcriptional coactivator in cardiac fibrosis. Elucidation of these different pathways promises the identification of new targets for specific inhibition of not only cardiac fibroblast proliferation and migration but also can be translated to the development of other diseases driven by EPAC1 expression, such as diabetics, cancer and fibrosis of other tissues.

Furthermore, all of our experiments were carried out *in vitro*. Therefore, Future works need to be carried out in order to test the importance of the mechanisms described here in more complex models, for example in myocardial tissue slice models or *in vivo*.

6.3 TRANSLATIONAL IMPLICATIONS

One of the major findings presented in this is novel mechanism regulating EPAC1 expression levels in response to cAMP levels in cardiac fibroblasts. The translational potential of these findings should be considered, since changes in EPAC1 expression as well as activity have been implicated in tissue fibrosis. Studies in mice demonstrated that EPAC stimulation not only decreased tissue fibrosis, but also suppressed cardiac remodelling and infarct size after MI (Surinkaew, Aflaki et al. 2019). Consistent with this, a number of published investigations demonstrated that EPAC1 expression in cardiac fibroblasts is down regulated in response to profibrotic agents (Basoni, Nobles et al. 2005, Yokoyama, Patel et al. 2008, Insel, Murray et al. 2012), implying the protective roles of EPAC1 during cardiac fibrosis. The recent development of novel non-cyclic EPAC agonists may therefore have therapeutic potential as a treatment to limit cardiac fibrosis (Parnell, McElroy et al. 2017). However, it should be noted that EPAC1 signalling in cardiomyocytes has been implicated in promoting cardiac hypertrophy (Laurent, Bisserier et al. 2015, Lezoualc'h, Fazal et al. 2016, Laudette, Zuo et al. 2018). Therefore, more research is now needed to test the potential of EPAC1 targeting therapies for the treatment of cardiac fibrosis

We demonstrated an important role for HDAC activity in the cAMP-mediated repression of EPAC1 expression. This suggests that HDAC activity may be involved in the down regulation of EPAC1 levels that are often observed in response to pro-fibrotic stimuli. Importantly, during the past decade, it was demonstrated that HDACs play a role in the progression of cardiac fibrosis (Ellmers, Scott et al. 2007) and mediate fibroblast activation (Guo, Shan et al. 2009). Moreover, abnormal HDAC

activity has been observed in cardiac fibrosis (Korfei, Skwarna et al. 2015). Several lines of evidence demonstrate that HDAC inhibition can alleviate cardiac fibrosis (Gallo, Latronico et al. 2008, Eom, Cho et al. 2011) and HDAC inhibitor therapy has been reported to be an effective therapy for patients with cardiac fibrosis (Schiattarella and Hill 2015). Consistent with these findings, we showed that HDAC inhibition, in combination with YAP over expression, was able to rescue the inhibitory effects of cAMP signalling on EPAC1 expression. The implication is that the therapeutic effects of HDAC inhibitors during cardiac fibrosis may be due, in part, to the restoration of EPAC1 expression and, hence its signalling capacity. However, the effects of therapeutic elevation of EPAC1 levels in the heart need to be considered carefully and thoroughly researched before trials in patients. Several studies have reported elevated levels of EPAC1 expression in animal models of cardiomyopathy and patients with failing hearts (Ulucan, Wang et al. 2007, Metrich, Lucas et al. 2008). Likewise, EPAC1 knockout mice studies show that loss of EPAC1 is protective against pressure overload-induced cardiac dysfunction (Okumura, Fujita et al. 2014), while EPAC activation can promote cardiac hypertrophy (Laurent, Bisserier et al. 2015, Lezoualc'h, Fazal et al. 2016, Laudette, Zuo et al. 2018).

The work presented in this thesis also highlights the important role of MKL-SRF and YAP/TAZ-TEAD in the regulation of cardiac fibroblast proliferation. As such, these transcription factor complexes may represent attractive therapeutic targets to limit cardiac fibroblast proliferation. Consistent with an important role for MKL in promoting tissue fibrosis pharmacological MKL activation promotes myofibroblast differentiation that is often associated with tissue fibrosis (Velasquez, Sutherland et al. 2013). Furthermore, deletion of the MKL1 gene in mice reduces fibrosis and scar formation following MI or Angiotensin II treatment (Small, Thatcher et al. 2010). There is also evidence that the YAP/TAZ-TEAD complex plays an important role in promoting fibrosis. For example, YAP activation has been shown to promote the trans-differentiation of cardiac fibroblasts to myofibroblasts and in matrix remodelling of dilated cardiomyopathy (Jin, Zhu et al. 2018). Recently, investigations in rodents demonstrated that conditional deletion of LATS1 and LATS2 in cardiac fibroblasts, which normally repress YAP and TAZ activity, induced cardiac fibrosis in mice (Xiao, Hill et al. 2019). In Zebrafish models of cardiac injury, hearts with YAP mutants demonstrated lower collagen synthesis at seven days post-cryoinjury, implying important roles of YAP in scar formation during heart regeneration (Flinn, Jeffery et al. 2019). YAP knockout in primary rat cardiac cells revealed a fibroblast-specific role for YAP in regulating pro-inflammatory cytokines and chemokines, implying that YAP-TEAD-mediated gene expression may

also have crucial roles in promoting inflammation, which is also an important factor prompting the fibrotic response (Flinn, Jeffery et al. 2019).

Although transcription factors are traditionally thought to be challenging drug targets, pharmacological inhibitors of MKL-SRF (Evelyn, Wade et al. 2007, Sandbo, Kregel et al. 2009, Evelyn, Bell et al. 2010, Bell, Haak et al. 2013, Johnson, Rodansky et al. 2014) and YAP/TAZ-TEAD (Liu-Chittenden, Huang et al. 2012, Zhang, Lin et al. 2014, Wang, Zhu et al. 2016, Kaan, Sim et al. 2017, Smith, Sessions et al. 2019) have been described. Pharmacological inhibition of MKL may have beneficial effects since local delivery of novel MKL inhibitors has been shown to prevent scar tissue formation in a preclinical model of fibrosis (Yu-Wai-Man, Spencer-Dene et al. 2017). Pharmacological MKL inhibition has also been shown to reduce liver fibrosis via inhibition of type I collagen expression in hepatic stellate cells (Shi and Rockey 2017) and can prevent fibrosis in a murine model of skin injury (Haak, Tsou et al. 2014). However, more research is required to fully characterise the potential of MKL targeting therapies as some research point to important physiological and developmental role of the MKL-SRF complex in the heart. For example, deletion of MKL1 in the heart results in cardiac abnormalities, such as: extreme ventricular dilation and decreased cardiac output (Mokalled, Carroll et al. 2015). In recent years several pharmacological inhibitors have also been described that target the YAP/TAZ-TEAD complex. However, these have not yet been evaluated in the context of cardiac fibrosis. The data presented here demonstrates that YAP-TEAD inhibition reduces cardiac fibroblast proliferation and hence suggests potential therapeutic benefits. It is therefore important that the efficacy of YAP-TEAD inhibition in animal models of cardiac fibrosis is evaluated.

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